ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

B MICROBIOLOGY

VOL. 85 B. FASC. 1 FEBRUARY 1977

MUNKSGAARD COPENHAGEN

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Founded 1924

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Acta Pathologica et Microbiologica Scandinavica is a nonprofit-making scientific journal. *Since 1924, it has been published by the Scandinavian Societies for Medical* Microbiology and Pathology It appears in three sections Section A Pathology, Section B Microbiology, and Section C Immunology

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ACTA
PATHOLOGICA
ET MICROBIOLOGICA
SCANDINAVICA

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ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section B MICROBIOLOGY

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MUNKSGAARD COPENHAGEN

Published by the
Scandinavian Societies for Microbiology and Pathology

Distributed by Munksgaard
International Booksellers and Publishers, Ltd.
35 Nörre Sögade DK 1370 Copenhagen K, Denmark

PRINTED IN DENMARK
VALD. PEDERSENS BOGTRYKKERI A/S
[illegible]

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Acta path. microbiol. scand. Sect. B, 85 1–8, 1977

DEVELOPMENT OF GERMFREE ANIMAL CHARACTERISTICS IN CONVENTIONAL RATS BY ANTIBIOTICS

Bengt E. Gustafsson and K. Elisabeth Norin

Department of Germfree Research, Karolinska Institutet, Stockholm, Sweden

Gustafsson, B. E. & Norin, K. E. Development of germfree animal characteristics in conventional rats by antibiotics. Acta path. microbiol. scand. Sect. B, 85 1–8, 1977

Conventional (CONV) rats were fed by stomach tube for five days with either benzylpenicillin, ampicillin, tetracykline, oxitetracykline, neomycin, bacitracin + neomycin, kanamycin or colistin. On the 2nd–3rd day all the animals developed one or several of the following symptoms or characteristics typical for germfree (GF) rats no coprostanol formation, no stercobilin production, GF pattern after gel electrophoresis of fecal supernatant and proteolytic activity in the feces. Under the same conditions succinylsulfathiazole or metronidazole had much less pronounced effects than the antibiotics. When clofibrate, acetylsalicylic acid or ferrous sulphate were administered the effects were none or negligible. The GF characteristics persisted for several weeks after the end of the administration of the drugs. In some instances this was the case up to 7 weeks, when the animals were contaminated by anal route with a suspension of the cecum contents from intact CONV animals. On the 2nd day after this treatment the GF characteristics had disappeared.

Key words Germfree animal characteristics antibiotics intestinal microflora coprostanol stercobilin.

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Received 14 II.76 Accepted 20 III.76

By feeding of antibiotics to conventional (CONV) rats several authors have produced germfree (GF) characteristics in CONV animals. Thus *van der Waaij* (16) found enlarged ceca in mice after bacitracin and streptomycin feeding for 7–9 days. To produce the same enlargement quantitatively as in GF rats, *Wiseman* (18) had to feed rats for more than 4 weeks with a mixture of bacitracin, streptomycin and nystatin. *Savage & Dubos* (13) found that penicillin, terramycin or kanamycin administration to mice caused a rapidly induced enlargement of the cecum. It is well known that administration of sulfa drugs or antibiotics to rats cause a change in the bile acid pattern of intestinal contents of CONV rats to that of GF rats (10 11 12). In children under GF conditions no coprostanol was present in the feces and feeding of oxacillin to normal children reduced the coprostanol content to near zero values (9). In GF rats stercobilin is not produced (8) and *Watson & Weimer* (17) demonstrated that antibiotic treatment of humans caused a noticeable decrease in the output of the urobilin group in feces and urine.

The present investigation was undertaken

TABLE 1 *Design of Experiments*

Series	Number of rats	Duration of administration (days)	Observation time (days)	Enema with fecal contents (on day)
I	33	5	5	
II	16	5	73	62
III	18	5	169	149
IV	14	5	50	

to study the initiation by some antimicrobial compounds of different GF characteristics, such as no formation of coprostanol (3) or stercobilin (8) a GF pattern after gel electrophoresis of fecal supernatant (7) and proteolytic activity in the feces (2) The influence on the bile acid pattern is reported separately (6)

MATERIALS AND METHODS

Design of Experiments

Four series of experiments comprising altogether 81 rats were performed (Table 1) In the first series the development of the GF characteristics was studied during 5 days administration of the drugs. Some of these animals were followed for several weeks to preliminary investigate the long term effects. In the second and third series both the short and the long term effects (max 5 months) were followed At the end of these experiments the establishment of a CONV microflora in the intestinal tract of the treated animals was studied by the administration of an enema with intestinal contents from intact animals. The fourth series was performed to evaluate the effects of different doses and preparations of metronidazole.

Animals

Male CONV rats of the Swedish (AGUS*) strain weighing about 275 gms at the start were housed individually in metabolism cages. These were placed in a rack and other CONV animals occupied the same room The personnel did not practice any isolation or aseptic measures. The animals were fed an autoclaved semisynthetic diet (5) water *ad libitum* and weighed once a week. Feces and urine were collected every 24 hours and frozen

After a pretreatment period of 5 days doses of substances to be tested were dispersed in 1 ml aliquots of water and given by stomach tube once a day for 5 days. In each series at least 2 rats were used for the test of one compound. Rats given water only and intact rats served as controls. The substances tested and the doses per 24 hours are given in Table 2

Germfree Characteristics

The shape, colour and consistency of the feces were recorded daily Stercobilin was determined as described earlier (8) The coprostanol to cholesterol ratio was calculated according to *Björkhem & Gustafsson* (1) Feces was diluted 1 2 with saline and centrifuged for ½ h at 35000 *g* Proteolysis was determined by incubating 0.2 ml of the supernatant with 5 ml 15 per cent gelatin solution for 2 h at 37 C The electrophoretic pattern of the supernatant was demonstrated according to *Gustafsson & Hellebro* (7)

Bacterial Counts

Total bacterial counts in feces were performed by incubating 10-fold serial dilutions anaerobically in thioglycolate medium (Difco) and on blood agar plates anaerobically and aerobically Enemas were given with 1 ml of a 10 ¹ dilution of cecum contents from 6 intact CONV rats of the same strain The dilution and handling of the intestinal contents were performed in a glove box with O free atmosphere, *i.e.* nitrogen passed through a column of reduced copper filings heated to 350 C. The copper filings were reactivated by a gas mixture of 10 per cent hydrogen and 90 per cent carbon dioxide

RESULTS

During the dosing of the animals those receiving the antibiotics had somewhat looser

* This rat strain of Long Evans origin has been reared under GF conditions at the Department since 1956 It has later been established at Laboratory Animal Center Carshalton, England and labelled AGUS

TABLE 2 *Germfree Characteristics During Administration of Drugs*

Compound	Proprietary name	Dose per rat/kg bodyweight	Number of rats changing from conventional to germfree fecal pattern/number of rats tested			
			Coprostanol	Stercobilin	Electrophoresis	Proteolytic activity
Penicillin	Benzylpenicillin Kabi	360,000 IU§	4/4	2/2	2/2	2/2
Ampicillin	Doktacillin Astra	725 mg	2/2	2/2	2/2	2/2
Tetracykline	Dumocyklin Dumex	160 mg	2/2	2/2	2/2	2/2
Oxitetracykline	Terramycin Pfizer	180 mg	2/2			
Neomycin	Neomycin Upjohn	160 mg§	6/6	2/2	2/2	2/2
Bacitracin + Neomycin	Bacimycin A-L	33,000 IU + 550 mg§	4/4	4/4	4/4	4/4
Kanamycin	Kanamycin Ferrosan	35 mg§	2/2	2/2	0/2	0/2
Erythromycin	Abboticin Abbott	360 mg§	4/4	3/3	3/3	3/3
Colistin	Colistinat Lundbeck	360,000 IU	2/2	2/2	2/2	2/2
Succinylsulfathiazole	Sulfadigesin Astra	360 mg§	3/4	1/2	0/2	2/2
Metronidazole	Flagyl Leo Elyzol Dumex	20 mg§ 100 mg†	1/14	1/14	0/14	2/14
Clofibrate	Atromidin ICI Pharma	90 mg	0/4			
Sorbitol	–	180 mg	0/4			
Acetylsalicylic acid	–	180 mg	0/4			
Ferrous sulphate		180 mg	1/2	1/2	0/2	0/2
Controls	–	–	0/21	0/10	0/10	0/10

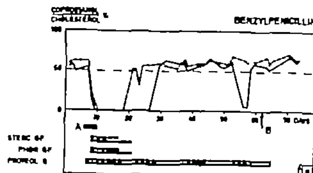
According to the Swedish drug list FASS

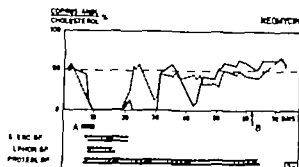
§ Doses used in the long term experiment series II.

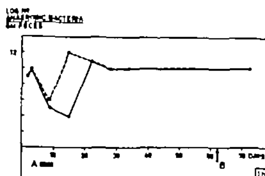
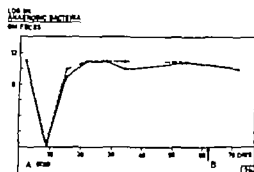
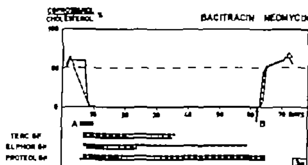
† Dose used in series IV

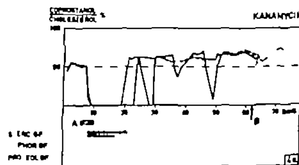
and more lightcoloured feces for a few days. The growth of the animals was good. One animal, originally dosed with kanamycin died with signs of peritonitis 2 days after administration of the enema of intestinal contents.

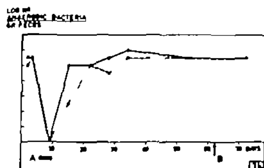
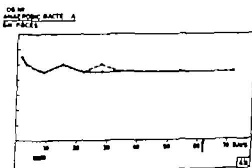
As is evident from Table 2 the feeding of the antibiotics caused the development of symptoms which are typical for GF rats within the period of administration for 5 days. Thus no coprostanol or stercobilin was formed, the electrophoretic pattern of the supernatant of diluted feces became GF like and proteolytic activity of the feces could be detected. These symptoms appeared in all the 28 rats given the antibiotics, with the exception only that kanamycin did not cause







Figs 1a–8a. Germfree (GF) characteristics in the feces of conventional (CONV) rats after treatment with antibiotics. Series II Coprostanol/cholesterol ratio (GF = 0) stercobilin absent—STERCO GF GF pattern of electrophoresis of fecal supernatant—ELPHOR GF and proteolytic activity—PROTEOL GF are listed A Dosing for 5 days. B. Enema with intestinal contents from intact CONV rats. Two rats – – – and —— were used for each drug

Figs 1b–8b Bacterial counts for the two animals in the corresponding Figs. 1a–8a.

changes in the electrophoretic pattern of the fecal supernatant or proteolytic activity. The effect during administration of succinylsulfathiazole and metronidazole was much less pronounced. This was still more the case for clofibrate, sorbitol, acetylsalicylic acid and ferrous sulphate, as only the last compound had some effect on coprostanol and stercobilin formation in one animal. The controls all showed CONV conditions.

In series II and III GF characteristics were present several weeks after the end of the ad

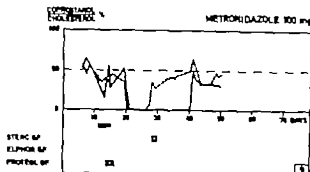


Fig 9 Text see Figs. 1a–8a.

ministration of the antibiotics. The results of the two series are corresponding why only those of series II are presented in Fig 1a–8a CONV conditions were not attained until after several weeks and in some instances not until the administration of an enema with intestinal contents from intact animals. This was the case for coprostanol formation after treatment with bacitracin + neomycin. The enema was also needed for regaining CONV conditions for proteolytic activity after dosing with benzylpenicillin neomycin, bacitracin + neomycin and erythromycin and in one animal with succinylsulfathiazole With this exception the latter compound and metronidazole had no long term effects in the doses given. In a separate series IV four proprietary compounds of metronidazole and the pure substance were tested The results were the same as in series II when the daily dose were kept at 20 mg per kg bodyweight. When the dose of the pure substance was increased to 100 mg per kg bodyweight the effect was somewhat more pronounced as is shown in Fig 9 There was, however a difference when compared with the antibiotics, as the effect on stercobilin and coprostanol formation appeared several days after the administration had terminated.

The counts of anaerobic bacteria in the feces have been listed in Figs 1b–8b There was a sharp drop in counts in some of the dosed animals on the 6th day but all counts were again within the range of the preexperimental period on the 23rd day The administration of erythromycin, kanamycin, succinyl sulfathiazole, metronidazole as well as water had a very slight or no influence on the number of anaerobic bacteria. The pattern of the number of aerobic bacteria in the tests performed was very much the same as for the anaerobic bacteria.

DISCUSSION

The administration to rats of broad spectrum antibiotics and also of benzylpenicillin was followed by symptoms which are characteristic for GF animals. The recorded changes were no formation of coprostanol or stercobilin a GF pattern after gel electrophoresis of fecal supernatant and proteolytic activity in the feces. A GF bile acid pattern was also induced in some animals (6) Benzylpenicillin was given in a dose per kg bodyweight equivalent to the therapeutic dose in humans. For erythromycin and for bacitracin + neomycin 12 fold human doses were used to get initial strong influences on the intestinal flora. The rats received the other drugs in daily amounts equivalent to 2 times or more the human therapeutic doses.

Metronidazole is active against anaerobic bacteria and has been proposed for the management of anerobic infections (15) The transformation of cholesterol to coprostanol and bilirubin to stercobilin has been demonstrated in exgermfre animals contaminated with single strains of anaerobic bacteria (4 8) The effect of metronidazole in the dose 2 times the dose used in human for the treatment of *Trichomonas vaginalis* was, however low on coprostanol and stercobilin formation in the animals. When the dose was increased 5 times to that used for the treatment of anaerobic infections in humans (15) the effect was still negligible on stercobilin formation the electrophoretic pattern of the fecal supernatant and on proteolytic activity The effect on stercobilin and coprostanol formation was delayed until several days after the end of the administration These divergences from the pattern of the other substances tested might be due to the fact that almost all the easily absorbed metronidazole is excreted in the urine and very little may reach

the part of the intestinal tract harbouring a physiologically active microflora.

There was a transient drop to undetectable levels in the total counts of fecal anaerobic bacteria in the animals receiving neomycin or bacitracin + neomycin and some influence in this respect also after treatment with benzylpenicillin. No such changes were seen in the other animals. The total bacteria counts do not, however mirror the pertinent changes in the flora. There was for example no decrease in the number of bacteria after dosing with erythromycin, but all four of the recorded characteristics appeared. *Sutter* & *Finegold* (14) observed the same high total counts of anaerobic bacteria in the feces of humans after treatment with cephalexin, cyclacillin and clindamycin as during the pretreatment period. The drop to zero of the number of anaerobic bacteria in the animals treated with neomycin is somewhat puzzling, as it is generally supposed that anaerobic bacteria like *Clostridia* and *Bacteroides* are resistant to neomycin. In the same samples there was no growth of aerobic bacteria. The effect of neomycin under these experimental conditions might be due to interaction with factors controlling the microbial ecology. These factors are at present not too well understood.

A significant result of the study is the long duration of the effect of 5 days administration of the drugs. In some animals, GF characteristics were not compensated until a normal intestinal flora had been established by a rectal infusion with intestinal contents from intact animals given 7 weeks after the end of the antibiotic treatment. This was also the case when benzylpenicillin was given in a dose equivalent to that for therapeutic use in humans. The pertinent microorganisms might not easily be reestablished in the animals, as they must have been abundantly present among the CONV animals housed in the same room and close to the treated animals.

The consequences of these findings for the use in humans of the compounds tested is influenced by the interpretation of the physiological and metabolic significance of the GF characteristics. The study demonstrates, however that rather simple biochemical methods can be used to judge whether a "normal" i.e. normally functioning intestinal flora is present or not. This might be of importance for practical reasons at least until more reliable methods have been introduced for the isolation, identification and definition of which organisms should constitute a "normal endogenous" microflora.

This work was supported by grants from the *Swedish Medical Research Council* (16X 205 206)

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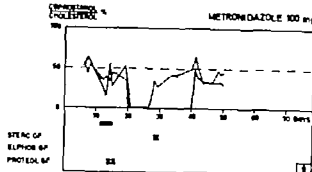


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Acta path. microbiol. scand. Sect. B, 85 9–13 1977

CROSS-REACTIVITY OF *BACTEROIDES FRAGILIS* O ANTIGENS

Tor Hofstad

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Hofstad, T Cross-reactivity of *Bacteroides fragilis* O antigens. Acta path. microbiol. scand. Sect. B 85 9–13, 1977

The cross-reactivity of O antigens prepared from *Bacteroides fragilis*, other *Bacteroides* species and from *Fusobacterium* has been examined by indirect haemagglutination and inhibition of haemagglutination. Fifteen of 20 *B. fragilis ss. fragilis* strains showed O-antigenic cross-reactivity with one or more of the test strains of *B. fragilis ss. fragilis* NCTC 9343 Lille E 323 and SBL B55. The same applies also to 3 strains classified as *B. coagulans B. hypermegas* and *B. putredinis*. The multispecificity of *B. fragilis* O antigens is pronounced Test systems for demonstration of 9 specificities, all harboured by one or more of the 3 test strains, have been worked out

Key words *Bacteroides fragilis* O-antigenic specificity O-antigenic cross-reactivity lipopolysaccharides.

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Received 10. [illegible].76 Accepted 25. [illegible].76

O-antigenic specificity is present in *Bacteroides fragilis* (5) Using indirect haemagglutination and inhibition of haemagglutination, six antigenic determinants, or factors, were detected in three cross-reacting strains of *B. fragilis ss. fragilis* isolated in separate laboratories (strains NCTC 9343 Lille E 323 and SBL B55) Like the Salmonella O antigens, the O antigens of *B fragilis* constitute the antigenically active part of the endotoxic lipopolysaccharide (LPS) (4)

The possibilities exist that the six antigenic determinants, found in the three cross-reacting strains and numbered from 2 to 7 are not monospecific and that the strains contain in their LPS undetected antigenic determinants shared with other *B. fragilis* strains. If so, the O antigens of *B fragilis* are extremely multispecific and more related to each other than the O antigens of other bacterial species. Conversely it is possible that the three randomly chosen strains are all members of a group of antigenically related strains and, as such, not representative of the majority of strains within subspecies *fragilis*. That means that the multispecificity may be less than indicated by the previous study (5) and that the antigenic relationship of the O antigens is analogous to that of other bacterial genera or species viz. the O antigen of a single *B fragilis* strain may share antigenic determinants with some strains but not with others within the species.

In an attempt to answer these questions, the multispecificity and antigenic relationship of *B fragilis* O antigens were further studied by examining

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fied LPS (3) or whole microbial cells, using 9 volumes of cell suspension (OD 2.5 at 600 mμ) for absorption of 1 volume of undiluted antiserum.

RESULTS

Continued work with the previously elaborated test systems (cf. Table 3) showed that some LPS preparations used for sensitization of sheep cells or HA inhibition tended to give unspecific agglutination. The unspecific agglutinations could partly be avoided if washed whole micro-organisms instead of LPS were used for absorption of antiserum. The sensitizing activity of crude LPS and its capacity to inhibit the agglutination of sheep cells sensitized with purified LPS were examined in a series of experiments. The results showed that crude LPS sensitized sheep cells to agglutination in unabsorbed and absorbed antisera, and had the same inhibiting capacity as purified LPS.

Multispecificity of B. fragilis ss. fragilis *O Antigens*

Sheep erythrocytes sensitized with 150 μg/ml of NaOH-treated crude LPS from the randomly chosen ss. *fragilis* strains listed in Table 1 (except NCTC 9343, E 323, SBL B55) were agglutinated by antisera to NCTC 9343, E 323 and B55 as indicated in Table 2. The pre-immune sera contained antibodies to three of the LPS examined, but not in titres exceeding 10. Sheep erythrocytes sensitized with LPS from two strains (VPI 5807 and SBL B59) were agglutinated to high titres (≥ 160) in all three antisera, and LPS from two other strains (one own isolate, one isolate from SBL) sensitized the sheep cells to high HA titres in two of the three antisera (anti-B55 and anti-E 323). Rabbit antisera against VPI 5807 and B59 were produced, and purified LPS from these two strains examined for antigenic determinants shared with one or more of LPS-9343, LPS-E 323 and LPS-B55 by HA and HA inhibition in cross-absorbed antisera to all five strains. Three new shared antigenic determinants (factors 8, 9 and 10) were detected. The test systems for their demonstration is shown in Table 3 which also includes the systems for demonstrations of factors 2–7. In addition, the two new strains harboured O-antigenic determinants not shared with the four heterologous strains. The 9 O-antigenic factors are distributed as *follows* in the previously used *test strains*: NCTC 9343:2, 3, 5, 10; E 323:2, 4, 6, 9; B55:3, 4, 7, 8. Factors 3, 8 and 9 are present in strain B59, and factors 2 and 10 in strain VPI 5807.

Sheep cells sensitized with crude LPS from 5 of the 20 ss. *fragilis* strains were not agglutinated by antisera to NCTC 9343, E 323 and B55 (Table 2). Crude LPS from the remaining 15 strains, except VPI 5807 and B59, were *now* examined by *HA* inhibition in test system 2–10. Factor 8 was present in 5 strains; one of these contained also factor 9. Factors 2 and 4 were found in each of 2 other strains. LPS from the other 6 cross-reacting strains contained none of the 9 antigenic determinants.

The absorbed antisera corresponding to factors 2 to 10 were also examined for antibodies agglutinating sheep cells sensitized with crude LPS from the same 13 ss. *fragilis* strains, which were not identical to anti-2 to anti-10. Such antibodies were present in a few absorbed sera, but in low titres.

TABLE 2. *Titres of Antisera to NCTC 9343, Lille E 323 and SBL B55 against Sheep Cells Sensitized with Crude LPS from 20* B. fragilis ss. *fragilis Strains*

Antiserum	No. of LPS agglutinated to titres		
	≤20	40–80	≥160
Anti-9343	12	1	2
Anti-B55	6	5	4
Anti-E 323	7	4	4

Reactivity of LPS from Different Bacteroides *and* Fusobacterium *Species in Antisera to NCTC 9343, B55 and E 323*

Crude or purified LPS from the *Fusobacterium* and *Bacteroides* strains listed in Table 1 (except *B. fragilis* ss. *fragilis*) were

TABLE 1 *Microbial Strains Used for Preparation of Crude or Purified LPS*

B. fragilis ss. *fragilis*	NCTC 9343, Lille E 323, SBL B55, SBL B59, VPI 5807, 14 own isolates, 3 isolates from SBL
ss. *thetaiotaomicron*	Lille 59, ATCC 12290, VPI 5333
ss. *distasonis*	ATCC 8503
ss. *vulgatus*	ATCC 8482
B. coagulans	VPI 3476
B. furcosus	VPI 3255
B. hypermegas	VPI 2366 = ATCC 25560
B. melaninogenicus ss. *intermedius*	NCTC 9336, VPI 4197 = ATCC 25611, VPI 7570-A, B10*
ss. *melaninogenicus*	VPI 4196 = ATCC 25847
ss. *asaccharolyticus*	ATCC 25260, NCTC 9337
B. putredinis	VPI 4998-1
B. praeacutus	VPI 0217 1
B. serpens	VPI 0950
F. mortiferum	VPI 0473 = ATCC 9817
F. naviforme	VPI 4877
F. necrogenes	VPI 2368
F. nucleatum	ATCC 10953, Fev 1§, 8 own isolates
F. plauti	VPI 4145
F. russii	VPI 0307
F. varium	VPI 0499, ATCC 8501

ATCC American Type Culture Collection, Rockville, Maryland. NCTC National Collection of Type Cultures, Central Public Health Laboratory, London. Lille Institute Pasteur de Lille. SBL National Bacteriological Laboratory, Stockholm. VPI Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

* Own isolate

§ Received from Dr *S. E. Mergenhagen*, NIDR, Bethesda, Md, USA.

sorbed and absorbed antisera to NCTC 9343, Lille E 323 and SBL B55, and the distribution of factors 2 to 7 within the strains. Also the antisera were examined for antibodies to additional antigenic determinants.

2. The reactivity of crude or purified LPS isolated from other *Bacteroides* species and subspecies, and from species of *Fusobacterium* in the same antisera.

MATERIALS AND METHODS

Organisms

The strains examined (Table 1) were isolated from man by the author and were mainly from pus or faeces, or they were obtained from other laboratories or national culture collections. The author's isolates were identified and subspeciated according to the Wadsworth Anaerobic Bacteriology Manual (8).

Cultures were grown in 500 or 1000 ml screw-cap bottles filled to the top with an enriched fluid medium based on whale meat extract and proteose peptone (2) or tryptone and proteose peptone (3).

Isolation of LPS

Packed wet cells were extracted with 45 per cent aqueous phenol (9). The water phase was dialysed and freeze-dried (*crude LPS*) or LPS was purified from it by ultracentrifugation and treatment with RNAse and DNAse (5).

Serological Methods

Antisera were produced in rabbits by immunization with whole cells (1). Sensitization of sheep erythrocytes with NaOH treated crude or purified LPS, indirect haemagglutination (HA) and inhibition of haemagglutination were performed as described (3).

Test sera used for the demonstration of O-antigenic determinants by HA inhibition were prepared by absorbing the rabbit antisera with puri-

9343 can be nearly completely separated from precipitating carbohydrate antigen (4).

The results of the study indicate that a typing system for *B. fragilis* based on the demonstration of defined O-antigenic specificities by HA inhibition may be worked out. For that purpose a wider spectrum of O factors and, consequently, more type strains, have to be included. However, for the time being serological typing of *B. fragilis* is of no practical importance.

Because of the small number of strains included the results were not analysed for a possible relationship between certain O-antigenic specificities and virulence.

This study was supported by the *Norwegian Research Council for Science and the Humanities.*

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TABLE 3 *The Test Systems for Demonstration by HA Inhibition of the O Antigenic Factors 2–10 in* B fragilis

Factor	Test system	
	antiserum*	sensitizing antigen
2	Anti-9343 abs. B55‡	LPS-E 323
3	Anti 9343 abs. E 323	LPS-B55
4	Anti B55 abs. 9343	LPS E 323
5	Anti 9343 abs. E 323 + B55	LPS-9343
6	Anti-E 323 abs. 9343 + B55	LPS-E 323
7	Anti B55 abs. 9343 + E 323	LPS-B55
8	Anti B55 abs. 9343	LPS-B59
9	Anti E 323 abs. 9343	LPS B59
10	Anti VPI 5807 abs E 323	LPS-9343

* 8 agglutinating units of antiserum.
‡ Absorbed with LPS or whole microorganism

DISCUSSION

With respect to O antigenic specificity *B fragilis* ss. *fragilis* seems to represent a heterogeneous subspecies. Within the subspecies there are strains which do not cross-react through their O antigens, and strains having one or more O factors in common The O antigenic cross-reactivity of ss. *fragilis* thus seems to be analogous to that of genus *Salmonella* The O antigens of the three test strains were more related to each other than to the majority of other O antigen preparations examined

The findings of the O-antigenic specificities 3 and 10 in *B coagulans* VPI 3476 and *B hypermegas* VPI 2366 show that the *B fragilis* ss. *fragilis* O antigens are not restricted to this subspecies. Recent experiments have shown also that some O factors are shared with ss. *distasonis* and ss. *vulgatus*

The O antigenic multispecificity of the *B fragilis* LPS is evident and is possibly more pronounced than suggested by the present study The serological reactivity of bacterial O antigens is due to the presence in the polysaccharide part of the LPS macromolecule of oligosaccharide units in the range of di to hexasaccharides bearing one or more specificities and often appearing as repeating side chains The specificity in turn depends on the nature and mode of linkages of the sugar components constituting the oligosaccharides. The O-antigenic specificities of *B fragilis* are harboured in the polysaccharide moiety of the LPS complex (3) but beyond this the chemical basis of the serological reactivity is unknown. Repeating oligosaccharide side chains carrying the different O factors may exist, or the multispecificity may be due to the presence in the same LPS of oligosaccharide units which differ in their chemical composition and/or configuration. A remote possibility is that part of the serological reactivity is due to contamination of the LPS preparations with another macromolecular polysaccharide A high molecular weight cell surface polysaccharide antigen which reacts by precipitation and seems to be group specific (6) has been demonstrated in strains of *B fragilis* (7) Recent fractionation experiments have shown however that the phenol/water extracted O antigen of strain NCTC

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9	Anti E 323 abs. 9343	LPS-B59
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P per Chromat graphy

Acid hydrolysis was performed in sealed tubes with N H_2SO for 4 h or with 3 N HCl for 3 h. The H_2SO hydrolysates were neutralized by passage through column of Dowex 1 in the formate form and acid was removed from HCl hydrolysates by evaporation in acuum over NaOH pellets. Circular paper chromatography was run with n-butanol-pyridine-water (6 4 3) Sugars were stained with aniline hydrogen phthalate or sil er nitrate Hexosamines were also sought with the Elson-Morgan reagent of *Partridge* (11)

Gas Liquid Chromatography (GLC)

Samples hydrolysed in 0.1 N HCl t 100 C for 48 h (12) were neutralized with Amberlite IRA 410, HCO form, and the aldoses converted to alditol acetates by the method of *Sawardeker et al* (13) GLC was run in Perkin-Elmer 900 Gas Chromatograph with a flame ionization detector and fitted w th a glass column (0.20 x 180 cm) packed with 3 per cent ECNSS-M (w/w) on Gas-Chrom Q, 100-120 mesh (Applied Science Laboratories, State College, Pennsylvania) The flow of gas (N) was 30 ml/min, the column temperature was 180 C and the detector temperature 260° C.

Chemical Analyses

Protein was measured by the Folin-Ciocalteu phenol method (10) Bovine serum albumin was used as standard. O-acetyl groups were sought as described by *Hestrin* (2) Phosphorus was measured with the method of *Fiske & S bbarow* (1) modified by *Youngburg & Youngburg* (14)

A alytical Ultracentrif gation and Imm noelectrophoresis

These analyses were carried out as described earlier (9)

Serological Methods

Antisera against whole microbial cells were produced in rabbits (5) Gel filtration of antiserum was performed at room temperature (22 C) using 72 cm high column of Sephadex G-200 with an internal diameter of 2 5 cm. The column was eluted with 0 1 M tris-HCl, pH 8 0 containing 1 M sodium chloride, t a rate of 1.8 ml/cm²h.

Double diffusion in agar gel and *ring test precipitation* were carried out as previously described (4) *In complement fixation test* (3) two 100 per cent lytic units of complement and two units of amboceptor were used. Tubes containing 0.1 ml of serum dilution, 0.1 ml of antigen, and 0.2 ml of complement were incubated overnight t 4 C, after which 0.2 ml of a 1 per cent suspension of sensitized sheep erythrocytes wa added.

I hibition of bacterial aggl tination Two-fold dil tions of the polysaccharide in 0 2 ml of saline were prepared in tubes. To ach t be was added 0.2 ml of antiserum containing 8 agglutinating unit Following incubation at room temperature for 30 min, 2 drops (pprox. 60 µl) of a saline suspension of L 452 microorganisms, standardized to an optical density at 600 mµ of 1.9 were added to each tube The agglutination was read after 3 h at 3 C.

Methods to be used for *indirect haemaggluti ation* has previously been reported (5)

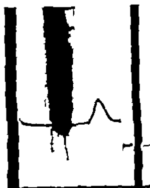


Fig. 1 Schlieren pattern of PS L452, 5 mg/ml in 0 05 M phosphate buffer pH 6 8 Exposure 73 min after speed is attained (59 780 rev/min, Spinco Model E, Rotor An-D)

RESULTS

The polysaccharide polymer hereafter referred to as PS L452 was prepared from two batches of L452 microorganisms. 2.4 g acetone-dried cells (Batch 1) were digested with 48 mg trypsin in 500 ml of buffer and 20 g washed, wet cells (Batch 2) were digested with 80 mg trypsin in 400 ml of buffer The supernatant fluid following centrifugation gave two agar precipitation lines against antiserum to whole L452 organisms a heavy line produced by PS L452 and a weak line representing a contaminating antigen.

PS L45? behaved in the same way as the previously isolated polymers PS L44 (6) and PS L49 (8) during the different steps of purification. Filtration through Sephadex G-75 separated the antigen from high-molecular-weight substances, and the DEAE-cel-

Acta path microbiol scand Sect. B 85 14–17 1977

COMPOSITION AND ANTIGENIC PROPERTIES OF A SURFACE POLYSACCHARIDE ISOLATED FROM *EUBACTERIUM SABURREUM*, STRAIN L452

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Hofstad T & Lygre H Composition and antigenic properties of a surface polysaccharide isolated from *Eubacterium saburreum* strain L452 Acta path. microbiol scand Sect. B 85 14–17 1977

A highly active surface antigen, reacting by precipitation and complement fixation, has been isolated from *E. saburreum* strain L452. The antigen is a polysaccharide polymer built up of galactose fucose and ribose.

Key words *Eubacterium saburreum* cell wall polysaccharide immunochemistry

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Received 25 viii 76 Accepted 25 viii.76

Eubacterium saburreum is a Gram positive anaerobic microorganism inhabiting the oral cavity of man Type-specific polysaccharide antigens have been isolated from two *E saburreum* strains, strain L44 (PS L44) (6 7 9) and strain L49 (PS L49) (8) PS L44 is a linear polysaccharide composed of partly O acetylated (1→6) linked β D-*glycero*-D *galacto*-heptopyranosyl residues (3) PS L49 is a heteropolymer containing chains of alternating (1→3) and (1→6) linked β D-glycero-D-galacto-heptopyranosyl residues, the latter being substituted with 6-deoxy-α D-*altro*-heptofuranosyl groups (4) Also PS L49 contains O-acetyl groups.

This report deals with the immunochemistry of a polysaccharide polymer isolated from *E saburreum* strain L452

MATERIALS AND METHODS

Cultural Conditions

E saburreum L 452 was isolated from human saliva. The organism was cultured in a fluid medium based on whale meat extract and proteose pepton, and supplemented with yeast extract, glucose and human or horse serum (8)

Extraction and purification Methods

The polysaccharide was prepared as previously described (6 8) In short acetone-dried or washed and packed cells were digested with trypsin (Trypsin 1 250 Difco) in a tris-HCl buffer pH 7 8 and the polysaccharide purified from the supernatant fluid following centrifugation of the digest (8 000 × g 30 min) by gel filtration (Sephadex G-75) ion exchange-(DEAE-cellulose) and affinity (hydroxylapatite Bio Gel HTP) chromatography

All antibody activity seems to be due to low molecular-weight antibodies, presumably IgG. The chemical structure of the polymers so far examined is unusual. PS L44 and PS L49 contain heptose which is a sugar commonly occurring in Gram-negative—but not in Gram-positive bacteria. Furthermore, PS L49 contains a deoxy sugar in the furanoid form not previously described.

The results of the present study show that PS L452 is a high-molecular-weight neutral polysaccharide antigen. The chemical composition is quite different from that of PS L44 and PS L49. Structural studies (*Hoffman et al.* manuscript in preparation) have indicated that fucose is in the furanoid form, which is extremely rare. In general, bacterial polysaccharides are built up of repeating oligosaccharide units. The simplest residue of PS L452 should be a trisaccharide of galactose, ribose and fucose. However, the gas chromatographic analyses do not support fully the postulation that the structure of PS L452 is like that of a polymer built up of repeating trisaccharide units. The capacity of PS L452 to inhibit bacterial agglutination shows that the antigen is exposed at the cell surface.

This study was supported by the *Norwegian Research Council for Science and the Humanities* and by *NIH Research Grant* No. DE 03821–01 from the *United States Public Health Service*.

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TABLE 1 *Chemical Composition* of the Polysaccharide Antigen PS L452 from* E. saburreum *Strain L452*

Cells	Yield mg	Ribose dry w %	Ribose mole %	Fucose dry w %	Fucose mole %	Galactose dry w %	Galactose mole %
Bath 1 acetone-dried 2 4 g	96	24 0	27 8	35 9	38 0	35.5	34 2
Batch 2 packed wet 20 0 g	163	22.9	?3 1	48.5	42.8	40 6	34 1

* The figures have not been corrected for water uptake during hydrolysis.

lulose column retained the contaminating precipitinogen while PS L452 went straight through the column.

PS L452 sedimented as a sharp peak by analytical ultracentrifugation ($S_{20}=2.36$) (Fig 1) and produced a single line situated on the kathodic side of the application point, on immunoelectrophoresis against antiserum L452

TABLE 2. *Serological Reactivity of the Polysaccharide Antigen PS L452 from* E. saburreum *Strain L452*

Precipitating activity*	0 98 µg/ml
Complement binding activity§	0 0078 µg
Minimal dose inhibiting agglutination†	12.5 µg

* Lowest concentration of PS L452 (2 fold dilution) giving positive ring test.

§ Lowest amount of PS L452 (2 fold dilution) giving maximum serum titre.

† Bacterial agglutination 8 agglutinating units of antiserum

Galactose, fucose and ribose were found by paper and gas liquid chromatography of acid hydrolysates (Table 1) Aminosugars were not detected The preparations of PS L452 were free from phosphorus, but contained small amounts of protein (4 3 and 4 2 per cent) O acetyl groups were not detected

The serological activity of PS L452 was examined against antisera to whole L452 microorganisms (Table 2) The polymer did not sensitize sheep erythrocytes to agglutination in L452 antiserum Fractionation of antiserum on Sephadex G 200 showed that all antibody activity to PS L452 was associated with the second protein peak (Fig 2)

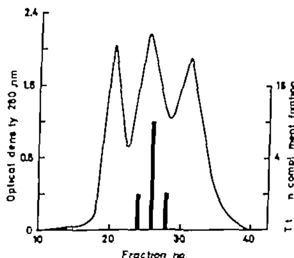


Fig ? Fractionation of 3 ml antiserum-L452 on Sephadex G-200 Fractions of 8.5 ml were collected. *Bars* complement fixation titres against PS L452 1 µg/ml.

DISCUSSION

Previous studies as well as the present report and preliminary examinations of other *E saburreum* strains strongly indicate that the organism has a carbohydrate polymer located at the outer part of the cell wall The polymer is a highly active hapten reacting in precipitation and complement fixation tests. Antisera against the hapten can be raised in rabbits by injection of whole microorganisms.

TABLE 1 *Fatty Acid Composition of* Neisseria elongata subsp. glycolytica *6171/75 Compared with* N. elongata M2*

Strain	Fatty acid‡													
	12:0	3-OH 12:0	14:1	14:0	3-OH 14:0	15:0	16:1	16:0	3-OH 16:0	17:1	17:0	18:2	18:1	18:0
6171/75	3.3	5.5	0.2	2.5	4.9	1.6	28.4	36.6	2.3	tr	0.5	0.8	11.5	1.0
M2	3.2	4.9	0.4	3.6	5.2	2.0	27.2	34.0	3.1	tr	0.4	1.3	13.0	1.0

* The two strains were grown, harvested, chemically prepared and gas chromatographed in parallel under strictly comparable conditions; the fatty acids were determined and quantitated as specified in previous reports on fatty acid composition in *Neisseriaceae* (9, 21, 22).

‡ The figures in front of colon indicate number of carbon atoms in the fatty acid chain, the ones after colon the number of double bonds. The symbol 3-OH indicates a hydroxy group in position 3 relative to the carbonyl group. The concentrations are given as percentages of the total fatty acid content. "tr" (trace) indicates less than 0.1 per cent.

TABLE 2. *Quantitative Streptomycin Resistance Transformation between* Neisseria elongata subsp. glycolytica *6171/75, the Type Strain M2 of* N. elongata *and* N. meningitidis

Experiment No.	Recipient strain (colony count/ml)	Donor strain	Transformants per ml	Ratio of interstrain to autologous transformation
1	Strain 6171/75	*N. elongata* M2	1.4×10^4	2.1×10
	(3.3×10^8)	*N. meningitidis* M1	1.1×10^3	1.6×10^{-2}
		Strain 6171/75	6.7×10^4	
2	*N. elongata* M2	Strain 6171/75	1.7×10^4	3.9×10^{-1}
	(9.4×10)	*N. elongata* M2	4.4×10^4	
3	*N. meningitidis* M1	Strain 6171/75	4.0×10^3	1.3×10^{-8}
	(6.8×10^7)	*N. meningitidis* M1	3.1×10^3	
4	Strain 6171/75	*N. elongata* M2	2.6×10^4	8.6×10^{-1}
	(1.6×10^8)	Strain 6171/75	3.0×10^4	
5	*N. elongata* M2	Strain 6171/75	2.9×10^3	1.7×10^{-1}
	(1.7×10^7)	*N. elongata* M2	1.7×10	

In experiments Nos. 1–3 transformation was performed in log phase fluid cultures (24). Short-term DNA exposure of lag phase cultures (8) was used in experiments Nos. 4 and 5.

Neisseria were included in the study as indicated in the tables.

The strain 6171/75 showed considerable variation and produced several different colony types (18). With a view to studying the colony type variation, the strain was grown on human blood agar prepared as described (7) and incubated at 33 C in humid atmosphere.

The formation of surface pellicles was studied in static cultures in Mueller-Hinton Broth (Difco) with 0.5 per cent yeast extract, incubated at 33 C and usually inspected daily for 4 days (Table 4). The following clones were isolated and subjected to further study:

SC-a. Comparatively large, corroding colony isolated from the first subculture (type 1, ref. 18).

SC-b. Smaller corroding colony.

Small N. Small, smooth, non-corroding colony isolated from the first subculture (type 2, ref. 18).

N-a and N-b. Smooth, non-corroding colony derived from clone SC-b.

N-c and N-d. Smooth, non-corroding colony derived from clone SC-a.

SC-c. Corroding colony derived from surface pellicle of stationary culture of clone N-d in broth.

2*

RELATIONSHIP OF *NEISSERIA ELONGATA* SUBSP *GLYCOLYTICA* TO OTHER MEMBERS OF THE FAMILY *NEISSERIACEAE*

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Bovre, K., Froholm, L. O., Henriksen, S D & Holten, E Relationship of *Neisseria elongata* subsp *glycolytica* to other members of the family *Neisseriaceae* Acta path microbiol. scand. Sect. B 85 18–26 1977

Neisseria elongata subsp. *glycolytica* strain 6171/75 is closely similar to the type strain of *N elongata* M2 as regards DNA base composition fatty acid content and electrophoretic mobility of two glutamate dehydrogenases, one of which showed a reaction of identity with the corresponding enzyme from M2 in double immunodiffusion in agar The strain showed genetic homologies with strain M2 in genetic transformation at a level suggesting species identity and with *N meningitidis* at a lower level. No affinity to *Moraxella* species or "false neisseriae" was demonstrated with the exception of a production of a few transformants in the 6171/75 recipient by DNA from *Kingella kingae* The strain showed the same pattern of associated variation of colony type fimbriation and competence in transformation as that found in other *Neisseria* and *Moraxella* species. After continuous subcultivation for some time some clones of the strain appeared to have lost the ability to produce acid from glucose

Key words *Neisseria elongata* genetic transformation competence fimbriae fatty acid composition glutamate dehydrogenase

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Received 9 viii.76 Accepted 9 viii.76

A new rod shaped member of the genus *Neisseria* was described in a preceding paper (18) The strain resembled *Neisseria elongata* in most respects but differed from the latter in that it produced acid from glucose gave a strong catalase reaction and that the consistency of agar colonies was distinctly different. According to the results obtained in transformation experiments, the strain appeared to be very closely related to the type strain M2 of *N elongata*. The strain was classified as a subspecies of *N elongata* and the name *N elongata* subsp *glycolytica* was proposed

The purpose of this paper is to report on additional studies of the properties of the strain and in particular of its relationship to other members of the *Neisseriaceae*

MATERIALS AND METHODS

In addition to the strain 6171/75 representative strains of species of *Moraxella* *Kingella* (17) false neisseriae (*N catarrhalis* and *N ovis*) and

TABLE 1 *Fatty Acid Composition of Neisseria elongata subsp. glycolytica 6171/75 Compared with N. elongata M2**

Strain	Fatty acid§													
	12:0	3-OH 12:0	14:1	14:0	3-OH 14:0	15:0	16:1	16:0	3-OH 16:0	17:1	17:0	18:2	18:1	18:0
6171/75	3.3	5.5	0.2	2.5	4.9	1.6	28.4	36.6	2.3	tr	0.5	0.8	11.5	1.0
M2	3.2	4.9	0.4	3.6	5.2	2.0	27.2	34.0	3.1	tr	0.4	1.5	13.0	1.0

* The two strains were grown, harvested, chemically prepared and gas chromatographed in parallel under strictly comparable conditions. The fatty acids were determined and quantitated as specified in previous reports on fatty acid composition in *Neisseriaceae* (9, 21, 22).

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TABLE 2 *Quantitative Streptomycin Resistance Transformation between Neisseria elongata subsp. glycolytica 6171/75, the Type Strain M2 of N. elongata and N. meningitidis*

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		Strain 6171/75	6.7×10^4	
2	*N. elongata* M2	Strain 6171/75	1.7×10^4	3.9×10^{-3}
	(9.4×10)	*N. elongata* M2	4.4×10^4	
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	(6.8×10^7)	*N. meningitidis* M1	3.1×10^3	
4	Strain 6171/75	*N. elongata* M2	6×10^4	8.6×10^{-3}
	(1.6×10^8)	Strain 6171/75	3.0×10^4	
5	*N. elongata* M2	Strain 6171/75	2.9×10^8	1.7×10
	(1.7×10^7)	*N. elongata* M2	1.7×10	

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N-a and N-b Smooth, non-corroding colony derived from clone SC-b

N-c and N-d Smooth, non-corroding colony derived from clone SC-a

SC-c Corroding colony derived from surface pellicle of stationary culture of clone N-d in broth

TABLE 3 *Semiquantitative Streptomycin Resistance Transformation of* Neisseria elongata *subsp* glycolytica *6171/75 with DNAs from Various Other Oxidase Positive Organisms**

Experiment No.	Donor DNA from	Net number of presumed transformants per plate
1	*N ovis* 199/55 *M bovis* ATCC 10900 *M urethralis* WM120 (ref 23) New Moraxella *Bijsterveld* (ref 1)	0
	K kingae T31 (ref 11)	35
	K kingae A1702	11
	K kingae 4177/66	6
	N flavescens ATCC 13120	>10 000
	N meningitidis M6	>10 000
2	*N catarrhalis* Ne 11 *M lacunata* ATCC 11748 *M nonliquefaciens* NCTC 7784 *M atlantae* A1922 (ref 9) *M osloensis* 5873 *M phenylpyruvica* 2863	0
	K kingae 4177/66	39

* In each experiment, identical aliquots of the recipient (clone SC-a) 10^7 to 10^8 colony forming units per plate of surface culture, were exposed to transforming DNA from the donors to be compared (10 µg) during growth at 33 C for 7 h, without DNase termination. The cultures were exposed to 50 µg of streptomycin per ml, added to the medium from below the agar The plates were incubated for a further 4 days and the number of resistant colonies was counted. The numbers are corrected for spontaneous mutants occurring without transforming DNA and in the presence of transforming DNA pretreated with DNase. Further details of this slightly modified long term DNA exposure have been reported (8) All donor strains have been employed in previous taxonomic studies (2 4 5 12 16) special references in the table)
N = *Neisseria* *M* = *Moraxella* *K* = *Kingella* (17) The designation *Neisseria* is temporarily used for "false neisseriae" (*N ovis* and *N catarrhalis*) and *Moraxella* for the *urethralis* organism.

Determination of DNA Base Composition

DNA of strain 6171 75 clone SC-a was prepared and its buoyant density in CsCl gradients measured in an analytical ultracentrifuge as previously described (5) These experiments were carried out in co-operation with Dr *Szybalski* McArdle Laboratory University of Wisconsin, Madison, Wisconsin *Cytophaga johnsonae* DNA served as density marker at density 1 6945 g/cm³ referring to the assumed density 1 710 of *Escherichia coli* K12 DNA. The average base composition, i.e., the moles per cent of guanine + cytosine (%(G+C)) was calculated from the buoyant density (g/cm³ = ρ) using the simplified formula %(G+C) = 1000 × (ρ 1 660) which is based on the value 50 %(G+C) of *E. coli* K12 DNA.

Fatty Acid Analysis

The applied cultural, chemical and gas chromatographic methods have been described in previous reports (9 21 22) The whole cell fatty acid composition of the strains 6171/75 SC-a and *N elongata* M2 (type strain) was compared, the procedures being applied strictly in parallel for the two strains (Table 1)

Examination of Glutamate Dehydrogenases

Cell free extracts were obtained by ultrasonic treatment of cells followed by centrifugation at 10 000 × g for 30 min (20) Electrophoresis of extracts was carried out in 1 2 per cent Difco Special Agar Noble in sodium barbital buffer pH 8.6, ionic strength 0 075 Glutamate dehydrogenase was localized by covering the gel with agar gel containing glutamate NAD or NADP or both, phenazine methosulphate and nitro blue tetrazolium as described (20) Ouchterlony double immunodiffusion tests were carried out in 1 per cent agarose in 0 85 saline at room temperature for 24 h. After drying the precipitate lines were stained with amido black (19)

Genetic Transformation

Quantitative streptomycin resistance transformation was performed by two methods 1) in log phase fluid culture as described by *Lie* (24) (Table 2) and 2) as the short term DNA exposure of lag phase cultures described by *Bøvre* & *Frøholm* (8) (Tables 2 and 4) Semiquantitative, long term DNA exposure transformation was performed as previously described (8) with few modifications as indicated in the legend of Table 3

Electron Microscopy

The methods used have been described previously (7 13)

RESULTS

Instability of Acid Production from Glucose

When strain 6171/75 was retested after subcultivation for some time acid production from glucose could no longer be observed Three clones representing different colony

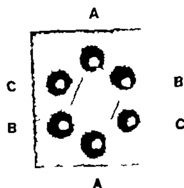


Fig 1 Double diffusion pattern of anti-glutamate dehydrogenase serum tested against crude extracts from strain 6171/75 *N elongata* and *N meningitidis* Centre well serum against NADP-dependent glutamate dehydrogenase from *N meningitidis* M6. A *N elongata* subsp. *glycolytic* 6171/75 B *N elongata* M2. C *N meningitidis* M1

types (types 1 2 and 4 as previously described (18)) which had been under continuous subcultivation since the isolation of the strain, were tested in several different glucose-containing media, but no acidity developed within 14 days. Subsequently an ampoule of freeze-dried culture prepared shortly after the isolation of the strain, was opened and tested the culture thus obtained showed slow production of weak acidity as before. The time of occurrence of the change and the nature of the latter are not known.

DNA Base Composition

The moles per cent (G+C) of DNA from strain 6171/75 was found to be 53.5 which is very close to the value of *N elongata* M2 (53.0 per cent ref 12) The value is by far higher than that applying to any known *Moraxella* species (5 9 23)

Fatty Acid Composition

As shown in Table 1 the fatty acid patterns of strain 6171/75 and *N elongata* M2 are almost identical. According to our experience such degree of similarity is a strong indication that the two strains belong to the same species (9 11 21)

Glutamate Dehydrogenase

Electrophoresis of extract from 6171/75 revealed that the strain had two glutamate dehydrogenases, one NAD-linked and one NADP linked The former migrated towards the anode and covered a distance equalling 105 per cent of that of the NAD-linked glutamate dehydrogenase from *N meningitidis* (20) The NADP linked enzyme migrated towards the cathode to about 40 per cent of the mentioned distance. These results are in excellent agreement with those obtained from three strains of *N elongata* (19)

Immunodiffusion was performed using antiserum against the NADP linked glutamate dehydrogenase from *N meningitidis* M6 and extracts from strain 6171/75 *N elongata* M2 and *N meningitidis* M1 (19) A reaction of partial identity between strain 6171/75 and *N meningitidis* was found, the latter forming a spur over the precipitation line of the former There was a reaction of identity between strain 6171/75 and *N elongata* M2 (Fig 1)

Affinities in Genetic Transformation

The results of quantitative transformation (Table 2) show an affinity between strain 6171/75 and *N elongata* M2 (inter to intra strain transformant ratios from 1 7 × 10⁻¹ to 8.6 × 10⁻ in the two directions) which strongly indicates species identity of the two strains (see also ref 18) The results of long term DNA exposure (without DNase termination) of strain 6171/75 (Table 3) show transformation incompatibility with donors of recognized species of *Moraxella*, false neisseriae" and some other organisms, but a distinct affinity to *N flavescens* and *N meningitidis* This confirms the very close relationship of 6171/75 to *N elongata* M2, which previously was found to belong genetically to the genus *Neisseria* (4 12) It is also worth noticing that *Kingella kingae* (*Moraxella kingae* ref 17) DNA appears to elicit a few transformants in the 6171/75 recipient, which may represent the first hint of a genetic relationship between the former species and

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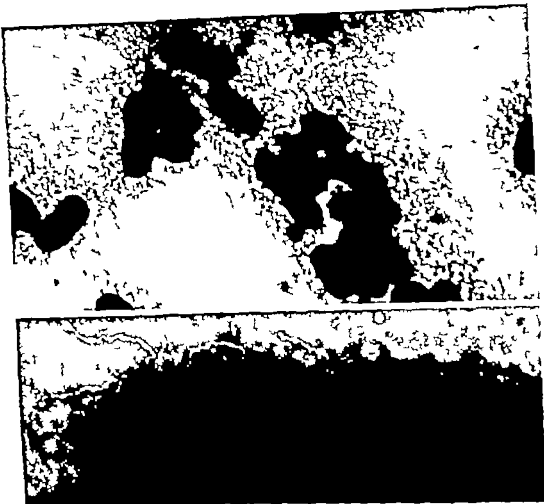


Fig. 3 Parallel preparation similar to Fig. 2 of the corresponding N-c variant. Upper 10,000 × lower 50,000 ×

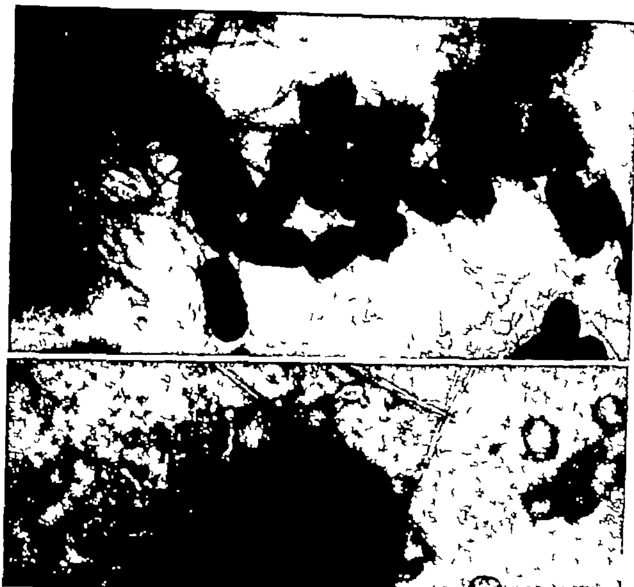
be straight, though with some distortions, and to be arranged in side-to-side clusters. The diameter of the individual filaments is about 4 nm, while the centre to-centre distance of parallel filaments is 6 to 8 nm.

The SC-a cells as well as N-c cells possess cell wall protrusions in the form of slender band-like structures of irregular outline. These structures may also occur freely dispersed and may assume the form of larger or smaller globules.

Cells from the SC-c and N-d clones were also studied and gave results similar to those obtained with the SC-a and N-c cells, respectively. The cells of the clone Small N which only showed a trace of corrosion and an intermediate degree of genetic transformation competence were either non-fimbriated or possessed only single fimbriae (Table 4).

Association of Genetic Transformation Competence, Colony Type Variation and Fimbriation

As presented in Table 4, the strain 6171/75 shows colony type variation and other cultural variation (pellicle formation) associated with fimbriation and genetic transfor



Figs 2 and 3 Electron micrographs of *N. elongata* subsp. *glycolytica*.
Fig 2 N elongata subsp. *glycolytica* 6171/75 SC-a, 15 h blood agar growth suspended in 0.8 per cent sodium silicotungstate pH 7 (7 13) Upper 10 000 × lower 50 000 ×

other members of *Neisseriaceae* Several experiments (not tabulated) using *Moraxella* false neisseriae and *K. kingae* as recipients and strain 6171/75 as donor of DNA were also performed, but were negative

Electron Microscopy of Colony Types

Fig 2 shows that the SC a clone of strain 6171/75 has numerous, mainly aggregated fimbriae which are evident at low magnification. By way of comparison Fig 3 shows that a granulated texture is characteristic of the surroundings of the cells of clone N-c studied by electron microscopy of negatively stained material. Using this technique, the N-c cells in general appear somewhat smaller than the SC-a cells (0.6–0.7 × 0.8–1.7 μm versus 0.8–0.9 × 1.1–1.8 μm) and the staining substance appears to be more concentrated close to the cell wall of the N-c cells. Fimbriae were only occasionally detected in preparations of N-c cells while practically all SC-a cells carried several fimbriae The fimbriae of the SC-a clone seem to arise preferentially at polar or subpolar locations, to

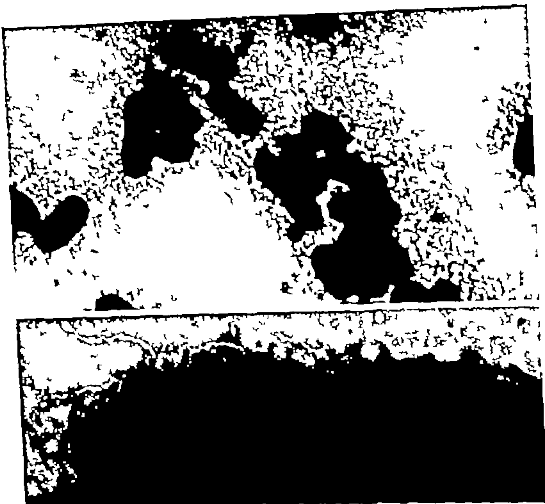


Fig 3 Parallel preparation similar to Fig 2 of the corresponding N-c variant. Upper 10,000 × lower 50,000 ×

be straight, though with some distortions, and to be arranged in side-to-side clusters. The diameter of the individual filaments is about 4 nm, while the centre to-centre distance of parallel filaments is 6 to 8 nm.

The SC-a cells as well as N-c cells possess cell wall protrusions in the form of slender band-like structures of irregular outline. These structures may also occur freely dispersed and may assume the form of larger or smaller globules.

Cells from the SC-c and N-d clones were also studied and gave results similar to those obtained with the SC-a and N-c cells, respectively The cells of the clone Small N which only showed a trace of corrosion and an intermediate degree of genetic transformation competence were either non-fimbriated or possessed only single fimbriae (Table 4)

Association of Genetic Transformation Competence Colony Type Variation and Fimbriation

As presented in Table 4 the strain 6171/75 shows colony type variation and other cultural variation (pellicle formation) associated with fimbriation and genetic transfor

TABLE 4 *Competence of Genetic Transformation Related to Fimbriation Agar Corrosion and Pellicle Formation in* Neisseria elongata *subsp* glycolytica *6171/75**

Clone/ cell line‡	(Parental clone)	Fimbriation†	Agar corrosion$	Pellicle formationδ	Competence (T/E ratio)ↄ
SC-a	Found in first subculture	+ +	+ + + +	+ + + +	1.5×10^{-4}
SC-b	SC-a, on plate, smaller colony		+ + + +	+ + + +	8.3×10^{-4}
Small N	Found in first subculture	(+)	(+)	(+)	4.8×10^{-6}
N a	SC-b on plate		(+)	(+)	$1 4 \times 10^{-7}$
N-b	SC-b, on plate		—	—	$<2 \times 10^{-8}$
N-c	SC-a, on plate	(—)	—	(—)	2.5×10^{-7}
N-d	N-c, on plate, larger colony	(—)	—	—	$<6 \times 10^{-8}$
SC-c	N-d occasional pellicle 10 days	+ +	+ + + +	+ + + +	$5 6 \times 10^{-4}$

* Quantitative short term DNA exposure as described (8) The transforming DNA was extracted from spontaneous streptomycin resistant mutants of 6171/75 SC-a and (for transformation of the recipients N-d and SC-c) of the N b variant.

‡ Nomenclature corresponding to previous reports on colony type variation in *Neisseriaceae* (7 8)

† + + most cells with detectable fimbriae (+) single fimbriae on occasional cells. (—) fimbriae only detected occasionally

$ + + + + distinct corrosion on the agar (+) a few pits of corrosion observed in heavy growth areas. — no corrosion observed.

δ + + + + distinct pellicle formation on fluid medium formed in 1–2 days. (+) weak pellicle formation in 4 days. (—) a weak transient surface film observed only once after 4 days of incubation. — no pellicle formed within 4 days.

ↄ T number of streptomycin resistant transformants per ml. E number of colony forming units exposed to transforming DNA

mation competence, as previously found in *M nonliquefaciens M bovis* and *K kingae* (6 7 8 13) Thus, cells from the spreading corroding clone SC-a were heavily fimbriated and highly competent whereas cells from the clone N c neither corroded the agar nor had more than occasional fimbriae and showed only a slight degree of competence as compared with those from the clone SC-a

DISCUSSION

The results obtained in this study reveal that the strain 6171/75 classified as *N elongata* subsp *glycolytica* certainly is very closely related to the type strain M2 of *N elongata* This is shown by the following findings the DNA base composition and fatty acid contents are practically identical they are in possession of two electrophoretically similar glutamate dehydrogenases, one NAD-linked and the other NADP linked, the latter enzyme from strain 6171/75 showing serological specificity identical to that of the M2 enzyme and the strains have a very high degree of genetic homology as demonstrated by transformation The degree of genetic homology is at a level corresponding to affinity levels found in strains of the same species (2 9 11 16) thus indicating that the strain 6171/75 is a true member of the species *N elongata*. In fact, the genetic affinity of this strain to strain M2 is higher than the affinities of the latter strain to several other isolates of this species (10)

If classification of the strain 6171/75 primarily were to be based upon genetic and chemotaxonomic evidence rather than on ordinary cultural biochemical characters, it would hardly have been given rank as a subspec es rather it would have been considered as a biovar of *N elongata*. If however it were to be based mainly on cultural biochemical characters, the strain might have been placed in a separate species, according to the traditional manner of classification of organisms belonging to this genus. It may be suggested that more extensive genetic and

chemotaxonomic studies also of other taxa in genus *Neisseria* might lead to further improvement of the classification of this group of organisms.

The fact that certain clones of the strain 6171/75 lost the ability to produce acid in glucose media after continued subcultivation indicates that some of the characters utilized in the classification and identification of *Neisseria* species are not as stable as desired. Irregular behaviour in diagnostic tests by another strain of *N. elongata*, including acid production from glucose, has been reported recently (11). It should also be borne in mind that asaccharolytic strains of *N. meningitidis* have been encountered (3).

The strain 6171/75 follows a pattern of association of colony type, pellicle formation, fimbriation and genetic competence as that found in several *Moraxella* species, *Kingella* and *Neisseria*. In this strain as in *N. elongata* M2 and in most of the other species examined, the fimbriated and competent colony type exerts marked corrosion on the agar surface. One exception appears to be *N. meningitidis* as competent variants of this species do not produce easily perceptible corrosion of agar (15), although the association of fimbriation, competence and pellicle formation has been demonstrated (14). The clone Small N of strain 6171/75 appears to have an unexpectedly high degree of competence considering the very low degree of fimbriation. In *Moraxella* the transformability of the SC variants always was 10^3 to 10^4 fold higher than that of the corresponding N variants. However, when different strains were compared, the competence of an N variant of one strain sometimes was found to be higher than that of an SC variant of another strain (6, 8). The fimbriae found in strain 6171/75 resembled those previously detected in *Moraxella*, *Kingella* and *Neisseria* species (7, 9, 13, 14).

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OXIDASE POSITIVE RODS FROM CASES OF SUSPECTED GONORRHOEA

A Comparison of Conventional Gas Chromatographic and Genetic Methods of Identification

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Bøvre, K. Hagen, N. Berdal, B. P. & Jantzen, E. Oxidase positive rods from cases of suspected gonorrhoea. A comparison of conventional, gas chromatographic and genetic methods of identification. Acta path. microbiol. scand. Sect. B, *85*: 27 37 1977

Genito-urethral specimens from 3260 women and 1170 men, with ailments suggestive of gonorrhoea, were examined for growth of oxidase positive rodshaped bacteria, as well as of gonococci. *Moraxella osloensis* was identified in 26 cases (0 64 per cent of women and 0 43 per cent of men) Three patients harboured phenylalanine negative (or weakly reacting) and tryptophan deaminase negative *M phenylpyruvica* and, in three cases, a *Flavobacterium* species was detected. Among six oropharyngeal specimen from patients suspected of gonorrhoea, two yielded growth of oxidase positive rods, *Kingella kingae* and *Neisseria elongata* respectively *N. gonorrhoeae* was isolated from 537 patients, i.e. 12.1 per cent of all cases. The isolates of oxidase positive rods were in most cases completely identified by streptomycin resistance transformation. On this basis, the diagnostic reliability of some morphological and cultural-biochemical tests and gas chromatography was examined. Gas chromatographic analysis of fatty acid and alcohol composition of whole cells proved distinctive of species defined genetically irrespective of confusing behaviour of some strains in other tests.

Key words Oxidase positive rods gonorrhoea gas chromatography genetic transformation.

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Received 28.viii.76 Accepted 6.ix.76

Taxonomic studies of *Neisseriaceae* by genetic methods have resulted in changed species concepts of oxidase positive rods (3 6, 9 10 12, 13, 16 17 18, 19 24) With a view to future research on the medical role of these micro-organisms it is important to collect data on their distribution in accordance with the new taxonomy In the present study their occurrence in conditions suggestive of gonorrhoea is examined. Morphological and cultural-biochemical tests and gas chromatography of cellular components as identification methods are evaluated versus affinities in genetic transformation.

MATERIALS AND METHODS

Source of New Isolates

Specimens from 4436 patients with clinical symptoms requiring cultivation of gonococci were simultaneously examined for growth of oxidase positive rods. The material comprised all speci-

mens of this type received in the Department of Microbiology University Hospital Tromsø during three months and two weeks in the winter of 1973–1974 Samples from 4430 patients (3260 women and 1170 men) were of cervical and/or urethral origin in six cases the investigation was performed on oropharyngeal specimens. In addition one isolate of oxidase positive rods from urine (pure culture more than 10^5 bacteria per ml) was included in the identification experiments.

Reference Laboratory Strains

Most of the strains serving as genetic and phenotypic references (Tables 1–4) were recently used in a study of *Moraxella atlantae* and *M phenylpyrouvica* (6) Two strains of *Flavobacterium meningosepticum* NCTC 10585 and NCTC 10587 were included in order to facilitate the diagnosis of three isolates not belonging to *Neisseriaceae*

Cultivation and Identification Procedures

The specimens were transported to the laboratory on swabs in Stuart medium, and primary cultivation was performed on non-selective heated blood agar as well as on Thayer Martin (27) medium (GC Agar Base (BBL) 36 g, Hemoglobin Powder (BBL) 10 g IsoVitalex Enrichment (BBL) 10 ml colistin, 7 5 mg vancomycin, 3 mg, distilled water to make 1000 ml pH 7 2–7 4) The culture plates were incubated for two days at 37 C in CO_2 incubators (National Meinicke Co with 10 per cent CO_2 and high humidity)

Isolates of oxidase positive rods were examined as follows The genetic affinities versus reference strains were tested by means of short term DNA exposure transformation of streptomycin resistance (3 5) Isolates not identified in this manner were examined by the more sensitive long term transformation technique (3 5) by which inter-species affinities were estimated Phenotypic characterization and comparison with laboratory strains followed the procedures proposed as minimal standards for the description of new taxa in the genera *Moraxella and Acinetobacter* (11) including more recent specifications and additions (6) Antibiotic sensitivity tests were performed by dilution of the drug in heated blood agar medium. The gas chromatographic and associated cultural and chemical procedures were as previously developed (20 22) with recent modifications (6)

RESULTS

Incidence and Species Distribution of Isolates

Oxidase positive rodshaped bacteria were detected in specimens from 34 patients, *i.e* from 0 77 per cent of those examined for gonorrhoea By way of comparison 537 patients (*i.e* 12 1 per cent) were positive for *Neisseria gonorrhoeae* In three patients both oxidase positive rods and gonococci were detected. In 26 cases (0 64 per cent of women and 0 48 per cent of men) *M osloensis* was grown from genito-urethral samples (*strains* T1 T26) Prior to sampling 11 of these 26 patients had received antibiotics (penicillin or tetracyclines) Genito-urethral isolates from three patients were *M phenylpyrouvica* (T28–T30) and from three patients the isolates were considered to belong to *Flavobacterium* (T33–T35) In two cases, the oropharyngeal specimens yielded oxidase positive rods *Kingella kingae* (12 17 19) labelled T31 and *N elongata* (T32) The strain obtained from urine was identified as *M osloensis* (T27) The data leading to identification are given below

TABLE 1 *Identification of New Isolates of* Moraxella osloensis, Kingella kingae *(19) and* Neisseria elongata *by Streptomycin Resistance Transformation**

New isolates	Ratios of interstrain to autologous transformation with reference recipient or donor			
	M osloensis A1920 or 5873	*K kingae* 4177/66	*N elongata* M2	7823/71
T1–27	35–93 %			
T31		47 %		
T32			5 %	73 %

* Short term DNA exposure (3 5)

Genetic Affinities

As shown in Table 1 the isolates T1–T27 reveal ratios of interstrain to autologous transformation with type or reference strains of *M osloensis* ranging from 35 to 93 per cent This range is normal for members of this species, as shown previously (2 9) Likewise the isolate T31 has intraspecies affinity to the type strain of *K kingae* (17) as expressed by the ratio 47 per cent. Strain T32

TABLE 2. *Genetic Relations of the New Isolat T28–T30 Assessed by Transformation of Spec of Neisseriae* ae

Exp. no	Recipient	Transformant numbers per plate with DNA donors used I parallel										
		M. onliq	*M. osloensis*		*M. atlantae*		*M. phenylpyruvica*					
		7784	A1920	5875	5118	A279	863	752/52	9413	T28	T29	T30
1	*M. nonliquefaciens* 7784		80	40						8	18	2
2	*M. osloensis* 5875	70			134	145	78			46	109	18
3	*M. atlantae* A1922							9	0	0	0	0
4	*N. catarrhalis* Ne 11		380	117	278	192	322			180	264	166

Long-term DNA exposure 3 5) *M* = *Moraxella*, *N* = *Neisseria* (both *Moraxella* (18) and *Branhamella* (14) have been proposed as genus name of *N. catarrhalis*). The affinities between the reference species are low or apparently absent if determined by w y of quantitati short-term DNA exposure transformation (5 6, 10) I Exp. nos 2–4 the T28–T30 donors show affinities to the recipients of almost the same degree as that of the *M. phenylpyruvic* donors; I Exp. no 1 the activities relati to *M. osloensis* donors are the same as those show by *M. phe ylpyru ica* in previous studies (6, 10). Open spaces not tested.

TABLE 3 *Application of Some Conventional Identification Tests in* Moraxella osloensis *and* M Phenylpyrouvica *Reference Strains and New Isolates**

Species / Strains	Fusiform cells with penicillin	Poly-β-hydroxybutyrate inclusions	Growth in Hutner medium with acetate	Growth at 4 C	Nitrate reduction to nitrite	Urease production	Phenylalanine deaminase	Tryptophan deaminase	Growth stimulation by bile salts	Bile salt MIC g/100 ml	NaCl MIC, g/100 ml	Penicillin MIC U/ml
M osloensis												
A1920 (type strain)	+	+	+	—	—	—	—	—	—	4	3	0.5
5873	–	+	+	—	+	—	—	—	—	0.05	3	0.1
T6	+	+	—	—	+	—	—	—	—	3	3	0.01
T20		+	+	—	+	—	—	—	—	2	3	0.05
T1–5 T7–19 T21–27	+	+	+	—	+—2	—w2	—	—	—	2–5	3	0.01–0.1
M phenylpyruvica												
2863 (type strain)§	—	w	+	w	—	+	+	+	+	5	9	0.01
10 other strains§	—w3+1	—	—w2	w	+	+—2	+	+	+	5	4.5–9	0.01–0.05
T28	+	—	w	w	w	+	—†	—	+	5	6	0.01
T29	w	—	w	w	—	+	—†	—	+	5	7.5	0.01
T30	w	—	+	w	+	+	—†	—	+	5	7.5	0.01

* Methods as previously described (6 11) MIC = minimal inhibitory concentration in solid medium. + strong reaction In the column of bile stimulation, + indicates effect of all concentrations tested in the range 0.05–4 g/ml w weak or slow reaction (6) In the column of urease production w indicates reaction only shortly after isolation — negative if two or more symbols occur together the first shows the most frequent reaction, while the following reactions are accompanied by the number of deviating strains. For other identification data, see Tables 1–2 and 4

§ Strains previously studied in detail (6)

† *Control* experiments performed by Professor *S D Henriksen* University of Oslo revealed some phenylalanine deaminase activity particularly with strain T29



Fig. 1. Strain T15 after growth for 20 h on blood agar around depot of penicillin. Gram-stained. Note fusiform cells and retention of the stal (black cells or parts of cells), both typical findings in *Moraxella odorans* under these conditions (970 ×)

shows high intraspecies compatibility (73 per cent) with *N. elongata* 7823/71. The lower affinity of this isolate to the type strain M2 (ref. 13), 5 per cent, is compatible with the previously observed genetic heterogeneity of *N. elongata* (7).

The three isolates T28–T30 did not disclose intraspecies affinity to any competent recipient of oxidase positive species. These isolates were incompetent and could therefore only be used as donors in genetic transformation. Furthermore, since competence as yet has not been detected in laboratory strains of *M. phenylpyrouvica*, identity with this species could not be directly tested. Table 2 shows comparative transformation experiments using the three isolates as donors of streptomycin resistance by the sensitive long-term DNA exposure technique. A certain affinity towards various species of *Moraxella* and *N. catarrhalis* is observed, of a magnitude corresponding to interspecies relations within this group (3, 6, 10). The reactions are compatible with identification of the strains T28–T30 as *M. phenylpyrouvica*, but do not rule out a species not yet described, with similar interspecies relations.

The isolates T33–T35 did not disclose any affinity to *Neisseriaceae* in transformation.

Morphological and Cultural Biochemical Characteristics

Table 3 shows some phenotypic properties of the isolates T1–T30 and reference strains of *M. odorans* and *M. phenylpyrouvica*. Frequently the *M. osloensis* isolates appeared as cocci in primary cultures, microscopically indistinguishable from gonococci. The formation of distinctly fusiform cells (Fig. 1) during growth at low concentrations of penicillin (6) appears to be a rather common feature of *M. odorans*. Coccal *Neisseria* species do not form rodshaped cells if similarly exposed and so far strains of *M. nonliquefaciens*, *K. kingae* and *N. elongata* have been negative if thus exposed, resulting in a formation of long threads of uniform thickness. Neither this test nor tests for growth on mineral medium with acetate or tests for phenylalanine/tryptophan deaminase, however, divide the strains recorded in Table 3 in accordance with the genetic data and, accordingly, a distinction on this basis of the strains T28–T30 from *M. osloensis* is impossible. On the other hand, the ability of *M. phenylpyrouvica* to grow at 5° C as well as its salt tolerance appear to be uniform and unique and shared only by the strains T28–T30. Also, the genetic data are confirmed by the uniform presence of poly-β-hydroxybutyrate inclusions in *M. osloensis* and the growth stimulation by bile of *M. phenylpyrouvica*, including the three new isolates.

The biochemical reactions of the isolate T31 corresponded to the description of *K. kingae* (17). On the other hand, strain T32 deviated from some of the reported characteristics of *N. elongata* (1, 13). Thus, it was nitrate positive and produced a small amount of acid from glucose in Hugh and Leifson

TABLF 4 *Fatty Acid and Alcohol Composition of M*

Species§	Strain†	12 0	3-OH 12 0	14 1	14 0	3-OH 14 0	15 0	16 1
M osloensis	T1	–	2.7	tr	–	1.0	–	14.3
	T2	–	3.8	–	0.2	1.4	–	7.1
	T3	–	2.5	–	tr	0.9	–	6.9
	T4	–	4.1	tr	0.1	1.7	–	6.9
	T5	–	4.9	tr	tr	2.1	–	8.7
	T6	–	5.1	tr	tr	1.9	–	15.3
	T7	–	3.3	tr	tr	1.8	–	12.4
	T8	–	2.9	tr	0.1	1.1	–	16.5
	T9	–	2.9	–	tr	1.2	–	17.0
	T10	–	2.8	tr	0.1	1.2	–	16.7
	T11	–	2.5	tr	tr	0.7	–	19.0
	T12	–	2.4	tr	–	0.9	–	18.3
	T13	–	1.0	–	–	0.3	–	17.6
	T14	–	2.2	tr	tr	0.9	–	17.5
	T15	–	2.7	0.1	0.1	0.9	–	18.7
	T16	–	1.5	–	tr	0.6	–	14.6
	T17	–	2.9	tr	0.1	1.1	–	17.3
	T18	–	2.6	tr	0.1	1.1	–	16.9
	T19	–	2.4	0.1	0.1	1.0	–	17.9
	T20	–	4.1	tr	0.4	1.3	–	17.8
	T21	–	2.8	0.2	0.1	1.1	–	17.6
	T22	–	3.5	0.4	0.2	2.0	–	9.8
	T23	–	2.8	0.1	0.1	1.1		17.6
	T24	–	2.7	0.4	0.1	1.1	–	18.4
	T25	–	2.5	0.2	0.1	1.1	–	18.3
	T26	–	2.6	0.2	0.1	1.0	–	17.3
	T27	–	2.8	tr	tr	1.0	–	14.8
	A1920	–	4.3	tr	tr	1.6	–	10.5
	A1920		4.0	tr	0.1	1.5	–	9.7
	A1920	–	4.6	tr	0.1	1.6	–	10.7
	5873	–	2.8	0.1	0.2	1.2	–	9.3
	5873		4.7	0.2	0.1	1.9		10.0
	5873		4.8	0.2	0.1	1.9		9.1
	x̄‡		3.16	0.09	0.09	1.25		14.2
	σ‡		0.99	0.10	0.07	0.43		4.1
M phenylpyruvica	T28	0.7	15.1	0.9	2.0	0.4	tr	9.1
	T29	0.6	10.9	0.6	2.2	tr	0.4	12.3
	T30	1.0	14.3	1.0	2.6	0.2	0.4	7.0
	11865/52	0.6	13.5	0.3	1.0	tr	0.4	9.4
M atlantae	A279	1.7	11.4	–	1.5	1.7	0.3	1.6
K kingae	T31	4.8	2.8	6.4	34.4	2.4	0.5	20.4
	4177/66	3.2	2.9	5.7	23.0	2.2	1.1	32.8
N elongata	T32	3.2	4.4	1.1	5.1	4.4	5.9	21.4
	M2	3.0	3.8	0.3	4.1	3.9	5.6	24.2

...Isolates of Oxidase Positive Rods and Some Reference Strains

16:0	3-OH 16:0	17:1	17:0	18 Oalc	18:2	18:1	18:0	Scored as M. [illegible]	M. phenylpyr	M. atlantae
3.4	–	2.6	–	0.6	1.4	71.2	2.4	14	2	3
7.2	–	1.5	tr	0.5	1.2	67.3	8.6	10	4	2
6.4	–	2.0	–	2.8	1.4	67.4	9.8	10	4	2
7.9	–	1.5	0.5	0.4	1.1	63.3	12.5	9	3	3
6.9	–	0.9	0.1	tr	0.7	65.9	9.0	7	4	3
6.2	–	1.3	–	0.4	1.3	65.0	3.3	12	2	3
4.2	–	2.3	tr	0.7	1.7	69.7	3.7	14	2	4
3.2	–	2.8	–	0.5	1.8	68.8	1.9	15	3	2
3.3		2.7	–	0.5	1.6	68.3	2.0	15	2	2
3.0	–	..8	–	0.6	1.7	68.7	2.1	15	3	3
6.2	–	3.4	–	2.4	3.9	56.9	4.8	10	4	2
2.3		2.3	–	0.6	2.4	69.2	1.6	13	3	3
2.8		2.8	–	0.6	1.6	70.8	2.4	11	3	3
2.7		3.1	–	0.7	1.7	69.1	1.9	15	3	2
3.4		2.7	–	0.5	1.8	67.4	2.0	14	5	2
2.9	–	0.8	–	0.8	0.9	74.4	2.4	11	3	3
2.8		2.6	–	0.5	2.5	67.3	2.1	13	3	2
2.9	–	3.0	–	0.6	1.8	68.5	2.0	15	3	3
3.0	–	2.7	–	0.5	2.4	67.9	1.9	15	4	2
10.8		2.2	0.3	1.2	2.0	53.8	6.2	12	4	2
2.8	–	2.9	–	0.5	1.8	68.6	1.7	14	4	2
5.9		5.9	0.9	0.9	2.8	61.1	6.6	7	4	3
2.7	–	3.1	–	0.6	2.5	67.3	2.2	15	3	3
2.9	–	2.5	–	0.5	2.3	66.6	1.8	13	4	1
2.6	–	2.9	–	0.5	2.1	68.2	1.9	14	4	1
2.9	–	3.0	–	0.5	2.2	68.0	2.0	14	4	1
3.5	–	2.8	–	0.8	1.7	69.8	2.5	15	2	2
6.3		0.9	0.5	–	1.5	63.6	11.0	10	5	3
6.7	–	0.8	0.2		1.5	65.0	10.3	10	6	4
5.9	–	0.9	0.3		1.7	62.3	11.4	10	5	3
7.4	–	3.0	0.5	0.9	2.5	61.6	9.4	9	5	3
5.2	–	3.0	0.4	0.6	2.5	65.0	5.5	11	5	4
5.9		3.5	0.5	0.7	3.1	65.5	6.5	8	5	4
4.35		2.47	0.12	0.67	1.90	66.47	4.75			
2.09		1.02	0.22	0.56	0.64	4.04	3.57			
4.3	–	0.6	0.2	–	11.0	20.7	7.7	3	14	5
24.9		0.6	0.3		13.9	22.4	8.7	3	15	4
27.2	–	0.5	0.2		11.1	19.4	9.1	2	12	3
22.1		0.6	0.3	–	16.0	23.0	9.1	3	15	4
32.4		0.6	0.8	0.6	14.1	16.5	15.3	3	7	13
11.6		0.1	0.1		3.0	9.0	1.9	4	4	1
10.0		0.2	0.1		3.9	11.9	1.8	4	3	1
34.4	2.2	1.1	1.9	/	1.5	11.4	1.2	1	2	0
33.0	1.9	0.6	1.0	–	2.3	11.8	1.5	3	3	2

For explanations see the next page.

1 Acta path. microbiol. scand. Sect. B, 85, 1

TABLF 4 *Fatty Acid and Alcohol Compositi*

Species§	Strain†	12 0	3-OH 12 0	14 1	14 0	3-OH 14 0	15 0
M osloensis	T1	–	2 7	tr	–	1 0	–
	T2	–	3 8	–	0 2	1 4	–
	T3	–	2.5	–	tr	0 9	–
	T4	–	4 1	tr	0 1	1 7	–
	T5	–	4 9	tr	tr	2 1	–
	T6	–	5 1	tr	tr	1 9	–
	T7	–	3.3	tr	tr	1 8	–
	T8	–	2 9	tr	0 1	1 1	–
	T9	–	2.9	–	tr	1.2	–
	T10	–	2.8	tr	0 1	1.2	–
	T11	–	2.5	tr	tr	0 7	–
	T12	–	2.4	tr	–	0.9	–
	T13	–	1 0	–	–	0.3	–
	T14	–	2.2	tr	tr	0.9	–
	T15	–	2 7	0 1	0 1	0.9	–
	T16	–	1.5	–	tr	0.6	–
	T17	–	2 9	tr	0 1	1 1	–
	T18	–	2.6	tr	0 1	1 1	–
	T19	–	2.4	0 1	0 1	1 0	–
	T20	–	4 1	tr	0 4	1.3	–
	T21	–	2.8	0 2	0 1	1 1	–
	T22	–	3.5	0 4	0.2	2 0	–
	T23	–	2 8	0 1	0 1	1 1	–
	T24	–	2 7	0 4	0 1	1 1	–
	T25	–	2.5	0.2	0 1	1 1	–
	T26	–	2 6	0 2	0 1	1 0	–
	T27	–	2.8	tr	tr	1 0	–
	A1920	–	4.3	tr	tr	1 6	–
	A1920	–	4 0	tr	0 1	1.5	–
	A1920	–	4 6	tr	0 1	1 6	–
	5873		2 8	0 1	0 2	1 2	–
	5873	–	4 7	0 2	0 1	1 9	–
	5873		4 8	0.2	0 1	1 9	–
	x̄§		3 16	0 09	0 09	1.25	
	σ§		0 99	0 10	0 07	0 43	
M phenylpyruvica	T28	0 7	15 1	0 9	2 0	0 4	tr
	T29	0 6	10 9	0 6	2 2	tr	0 4
	T30	1 0	14 3	1 0	7 6	0.2	0 4
	11865/52	0 6	13.5	0.3	1 0	tr	0.4
M atlantae	A279	1 7	11 4	–	1 5	1 7	0 5
K kingae	T31	4 8	2 8	6 4	34 4	2 4	0 5
	4177/66	3 2	2 9	5 7	23 0	2.2	1 1
N elongata	T32	3 2	4 4	1 1	5 1	4 4	5 9
	M2	3 0	3 8	0 5	4 1	3 9	5 6

can be considered consistent with the identity *M phenylpyruvica* of these new isolates.

The fatty acid pattern of the isolate T31 is compatible with the genetically proved identity *K. kingae* and that of T32 with *N elongata*. The patterns of the isolates T33–T35 were almost identical, being characterized by branched fatty acids and several unidentified peaks by which they were clearly distinguished from *Neisseriaceae* (20, 21) The patterns were very similar to those of the reference *F meningosepticum* strains, and these isolates were tentatively allocated to the genus *Flavobacterium*.

DISCUSSION

This study indicates that *M osloensis* is the oxidase positive rodshaped micro-organism most frequently occurring in the human genito-urethral region. Still, it appears to be present only in few individuals. Any special association of *M osloensis* to these surfaces as a normal inhabitant or possible pathogen may therefore be considered rather uncertain. By way of comparison, the habitat of *M nonliquefaciens* (and *N catarrhalis*) in the nasal cavity of man is much more firmly established (4)

The diagnostic aspects of these findings are considered to be important in examinations for gonorrhoea. *M osloensis* often appeared in the primary cultures as coccal cells indistinguishable from *N gonorrhoeae* It was also clearly shown that *M osloensis* can be enriched on Thayer Martin medium. This represents a warning against the unqualified use of commercial Thayer Martin medium devices designed to be read only by growth of oxidase positive colonies without secondary cultural tests. Microscopy of the primary cultures is not sufficient.

Our results may be compared with findings in a similar study by *Kittner et al.* (23) These authors examined genito-urethral specimens from women and found moraxellae in 0 77 per cent of the cases. There was no clear-cut difference between patients from whom gonococci were isolated, patients with inflammatory signs but negative gonococcus culture and a control group of healthy women. In conformity with the results obtained by us, *Kittner et al.* noted that several of the isolates primarily were indistinguishable from *N gonorrhoeae* whether by colony morphology or microscopy Almost all their isolates were identified as *M nonliquefaciens* although they did not report the use of tests which are necessary to differentiate between *M nonliquefaciens* and *M osloensis* (24) That their isolates might have been *M osloensis* is consistent with rather high MIC$_{peni cilli n}$ values (0.25–1 U/ml) in the majority of their strains. Penicillin resistance of *M osloensis* has been observed also by other investigators (15) However the MIC values of almost all of our *M osloensis* isolates ranged at 0.05 U/ml or less.

The object of the second part of this study was to elucidate, for general purpose, how the diagnostic value of morphological and ordinary cultural-biochemical tests, as well as gas chromatography of whole cells, would perform in an unselected material of genetically identified oxidase positive rods. The results point to some apparently stable cultural-biochemical traits of high value in identification, particularly the low temperature growth and the salt tolerance of *M phenylpyruvica* (6, 25) The less specific, but uniform bile salt stimulation of this species (6, 25) and the formation of poly β hydroxybutyrate inclusions in *M osloensis* (24) are also of diagnostic importance. Other properties may be more variable and less distinctive than expected. This adds to the problems arising from the methodological irreproducibility of several of these tests (8) Even some key reactions of *Moraxella* species (24) may fail. One example is the observation of phenylalanine deaminase negative (or weakly reacting) and tryptophan deaminase negative *M phenylpyruvica*. The existence of such strains has been suggested (26) though never demonstrated as clearly as in the present study. In addition, these and other strains of *M phenylpyruvica* may grow on mineral

O/F medium. The latter property appeared only in later subcultures after isolation Fur thermore, this strain showed weak to distinct reactions in the phenylalanine and tryptophan deaminase tests. The isolates T33–T35 were glucose and maltose positive but varied with respect to sucrose acidification nitrate reduction and phenylalanine and tryptophan deamination.

As regards *M osloensis* the minimal inhi bitory concentrations (MIC) of penicillin (Table 3) were 0 05 U/ml in 22 of the new isolates, being 0 1 U/ml in three strains and 0 01 U/ml in two strains. Seventeen out of these 27 *M osloensis* strains (*i.e* 63 per cent) were primarily streptomycin resistant (MIC = 200–800 μg/ml) whereas the rest had MIC = 0 1–0 4 μg/ml

The *M osloensis* strains T1–T27 showed MIC values of colistin and vancomycin rang ing from 1 6 to 25 μg/ml for each antibiotic. Most strains were able to grow, usually at somewhat reduced rates, in the presence of 7.5 μg/ml of colistin and 3 μg/ml of vancomycin, which are the concentrations in the Thayer Martin medium. Correspondingly these strains were often clearly enriched on this medium in primary culture. The strains T28–T30 (*M phenylpyrouvica*) were sensi tive to colistin (MIC = 0 1 μg/ml) and resistant to vancomycin (MIC = 25 μg/ml) and consequently did not grow on Thayer Martin medium The behaviour of strain T32 (*N elongata*) was similar ($MIC_{colistin}$ = 0 1 μg/ml and $MIC_{vancomycin}$ = 7.5 μg/ml) The isolate T31 (*K kingae*) was resistant to both agents ($MIC_{colistin}$ = 25 μg/ml and $MIC_{vancomycin}$ = 100 μg/ml) and grew on the medium the same applied to the isolates T33–T35 ($MIC_{colistin}$ > 100 μg/ml and $MIC_{vancomycin}$ = 7.5–25 μg/ml)

Fatty Acid and Alcohol Composition

Table 4 shows the fatty acid and alcohol contents of the isolates T1–T32 and a num ber of reference strains. The strains T1–T27 identified genetically as *M osloensis* reveal quantitative and qualitative distributions of compounds similar to the reference strains of this species and distinctly different from all other strains of the material. The isolates T28–T30 group together with a strain of *M phenylpyrouvica* listed in the table, as well as with 10 other strains of this species examined previously (6) *M atlantae* is in cluded in the comparison (6) This species is very close to *M phenylpyrouvica* in terms of fatty acid composition, but its content of the wax ester alcohol n-octadecanol (18:0alc) serves to distinguish it from the latter organism (6) The strains T28–T30 do not show a resemblance to *M atlantae* greater than that of typical *M phenylpyrouvica* and the alcohol is absent With the recently provided information about the fatty acid composition of other *Moraxella* species (20) the results

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* Experimental details as previously described (20 22) with recent modifications (6) Key to fatty acid designations the figure in front of colon indicates number of carbon atoms in the chain, the figure after colon the number of double bonds the symbol 3-OH indicates a hydroxy group in position 3 relative to the carboxyl group 18 0alc denotes a straight chain saturated fatty alcohol (n-octadecanol) The concentrations are given as percentage of the total acid (plus alcohol) content. A few minor unidentified compounds have been excluded from the table. tr" indicates less than 0 1 per cent.

‡ Genetically defined entities (2, 6 9 13 17) or as regards *M phenylpyrouvica* a species concept formed from low or absent compatibility with other known species and some phenotypic traits presumed to be stable (6 10) *M* = *Moraxella* *K* = *Kingella* (19) *N* = *Neisseria*

† Strains labelled T are new isolates in the present study and allocated to species by genetic transformation (Tables 1–2) The other strains are reference strains recently employed in taxonomic studies (6 20) *M osolensis* A1920 and 5873 were examined pairwise in three independent experiments.

$ The mean value $\bar{x}$, standard deviation σ and the confidence interval $\bar{x} \pm \sigma$ have been calculated for each of the fifteen compounds of *M osloensis* (tabulated) and, as previously reported of *M phenylpyrouvica* and *M atlantae* (6) Score" indicates how many of the fifteen tabulated concentrations of a strain which fall inside the confidence intervals applying to each of the three species.

21 *Jantzen, E., Bryn, K. Bergan T. & Bøvre K.* Gas chromatography of bacterial whole cell methanolysates. VII Fatty acid composition of *Acinetobacter* in relation to the taxonomy of *Neisseriaceae*. Acta path. microbiol. scand. Sect. B, *83* 569–580, 1975.

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27 *Thayer J. D. & Martin, J. E.* Improved medium selective for cultivation of *N. gonorrhoeae* and *N. meningitidis*. Public Health Reports *81* 559–562, 1966.

media with acetate, a main characteristic used for identification of *M osloensis* (24)

The taxonomic utility of gas chromatographic fatty acid and alcohol analysis of whole cells has previously been evaluated in *Neisseriaceae* using genetic affinities as parameter (20 21) The present study of new isolates partly behaving atypically according to conventional tests, consistently revealed gas chromatographic patterns of high specificity in accordance with the genetic data. Thus, this method can be considered to be of an accuracy approaching that of genetic tests in identification of moraxellae and similar bacteria.

This study was supported by the *Norwegian Research Council for Science and the Humanities* grant, C.07 01 1 (to K.B)

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ation or acquisition of polymer bridges between the cell and the surface *attachment*. Third, the increase in cell number by cell division or additional apposition of cells from the suspension *accumulation*. In a previous communication (8) we have described the attachment of streptococci to the protein film on tooth enamel *in vitro*. In the present study an *in vitro* model system for bacterial sorption to glass has been evaluated. A strain of *Streptococcus faecium* was employed. It was chosen for the purpose of comparison, in ensuing communications, with oral bacteria reactive with oral fluids. In preliminary experiments, *S. faecium* was shown not to react with human saliva.

MATERIALS AND METHODS

Cells. A laboratory strain of *Streptococcus f.* was used. The organism was non-motile. It grew at 10 and 50° C, at pH 9.6, in the presence of 6.5 per cent NaCl and in 0.1 per cent methylene blue milk. It fermented arabinose but not sorbitol and produced ammonia from arginine. These characteristics are consistent with a species designation of *Streptococcus faecium* according to *Bergey's Manual of Determinative Bacteriology* (2). The bacteria were maintained in a semisolid thioglycollate medium, subcultured on blood agar and grown in Trypticase Soy Broth (TSB, *BBL, Cockeysville, Md., USA*) at 37 C under atmospheric conditions.

Thymidine incorporation. T 90 ml of fresh TSB was added 10 ml of an overnight culture of *S. faecium* in the same medium. After incubation for 2 hours, (6-^{3}H)-thymidine (23.4 Ci/mmol, 103 mCi/mg, *The Radiochemical Centre, Amersham, England*) was added to final activity of 1 μCi per ml. Following 45 min of incubation in the presence of radioactively labelled thymidine, the cells were harvested by centrifugation (10,000 × g, 15 min, 4 C), washed three times and resuspended in the buffer or salt solution to be used for the experiment.

Experimental proced e. After harvesting, washing, and redispersion of the cells, the absorbance (A) of the bacterial suspension was measured in Perkin-Elmer 124 spectrophotometer. In general, suspensions of A_{540nm} ~ 1.0 were prepared for the experiments. Two-ml portions of the test suspensions were distributed to 16 × 44 mm plastic test tubes, and soda-lime cover glasses (*Karl Hecht, Sondheim/Rhön, West Germany*), 12 × 12 mm, were added, one to each tube. The cover glasses were in a ertical position during incubation at room temperature under atmospheric conditions. On termination of the experiment, the cover glasses were removed by pliers and immersed for three seconds in the suspending medium without bacteria. The glasses were left to dry in a vertical position, standing on filter paper and supported by metal rack. The glasses with sorbed bacteria were then formamide-treated and assayed for radioactivity as described below.

Liquid scintillation counti g. When suspensions of labelled bacteria were to be assayed for radioactivity 50 μl aliquots were transferred to the scintillation vial. When labelled cells were dried onto or sorbed from suspension to cover glasses, the glasses with adhering cells were transferred to the vials. Five hundred μl of formamide was then added to each ial and allowed to act on the cells for 2 h at 37 C during continuous stirring (6). Finally each ial received 10 ml of a dioxane-xylene based scintillation liquid, and radioactivity was measured in a Packard Tri-Carb 2450 liquid scintillation counter. For each experiment, a standard curv relating scintillation counts to bacterial density was obtained. 10, 20 and 50 μl of the test suspension was pipetted onto cover glasses, left to dry, formamide-treated and counted as described. In the case of streptococci, A_{540nm} = 0.5 corresponds to total count of approximately 10^9 organisms per ml (8); thus scintillation counts could be transformed into bacterial cell numbers. All samples were prepared and counted in triplicate.

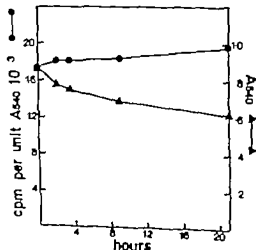


Fig. 1. Retention of (6-^{3}H)-thymidine in bacterial cell suspensions. At intervals during a 21-hour period, the suspensions were washed 3 times and resuspended prior to measurements of radioactivity and optical density.

SORPTION OF *STREPTOCOCCUS FAECIUM* TO GLASS

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Ørstavik, D Sorption of *Streptococcus faecium* to glass. Acta path. microbiol. scand. Sect. B, 85 38–46 1977

A method has been developed by which to study the sorption of *Streptococcus faecium* to soda lime cover glasses. Conditions were chosen to minimize the influence on sorption of bacterial polymer production, passive sorption being studied rather than attachment mediated by metabolic activities. Sorption of *S faecium* increased with increasing temperature (to 50 C) time and cell concentration but equilibrium apparently was not reached even after incubation for 8 hours or at a cell concentration of 3×10^{10} per ml. Sorption increased with solute molarity up to 0 1 M concentration of NaCl and KCl, indicating an effect of the electrical double layers on the apposition of cells to the glass surface. Desorption of bacteria could be obtained after multiple washings of the glasses in buffer or by the action of Tween 80, but not if sorbed bacteria were left in distilled water various salt solutions, urea, or in suspensions of unlabelled bacteria. It was concluded that sorption occurred as a result of chemical interactions between the glass and the cell surface. Tween 80 at a concentration of 1 per cent inhibited sorption to 26 per cent of buffer controls, 2 M urea was less effective and 1 M NaCl was without effect. It is suggested that hydrophobic interactions may be of importance in the binding of *S faecium* to glass.

Key words Sorption *Streptococcus faecium* glass.

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Received 6 viii 76 Accepted 10 ix 76

Various conceptual frames have been applied to interpret bacterial settlement and growth at interfaces. *ZoBell* (10) considered the concentration of nutrients and the stabilization of exocellular enzymes favourable for the growth of bacteria on solid surfaces *Marshall et al* (3) considered the initial sorption of bacteria to glass to be a reversible association resulting from physical, attractive forces Metabolic activities, i.e. the production of exocellular fibrillar material effected the subsequent irreversible attachment of these loosely sorbed cells. The attachment of *Streptococcus mutans* to glass may also be mediated by exocellular polymers produced by this organism (5 7) and the inference has been made that a similar mechanism is operative *in vivo* in the colonization of teeth by *S mutans* Yet another form of bacterial attachment is mediated by extraneous polymers present in the environment the protein film on tooth enamel surfaces promotes the attachment of certain oral streptococci (8)

Bacterial accumulation on a solid surface may be divided into three seperate phases. First, the immobilization of a bacterial cell on the surface *sorption* Second the elabor

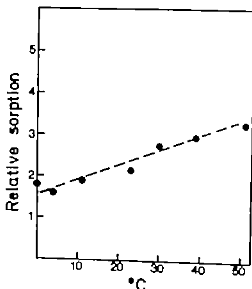


Fig. 4 Sorption of *S. faecium* to glass as function of temperature. Incubation for 40 min. Means of 3 measurements

tration. Bacterial suspensions ranging in density from A_{540nm} = 0.25 to 16.00 were prepared in phosphate-buffered saline (PBS 0.14 M NaCl, 0.01 M phosphate buffer pH 7.4). Cover glasses were incubated with the suspensions for 40 min. Sorption of bacteria increased up to the highest bacterial density tested, but tended to level out at the higher cell concentrations (Fig. 2).

Effect of incubation time. Sorption increased with increasing incubation time (Fig. 3). When the glasses were merely dipped into the suspension, a substantial number of organisms sorbed instantly. This initial, rapid rate of sorption fell abruptly when the glasses were left for longer periods of time; there appeared to be a steady and increasing decline in the rate of sorption up to 8 h. Sedimentation of bacteria in the suspension precluded observations beyond 8 h.

Area coverage. The almost linear relationship between bacterial cell density and the number of sorbed bacteria at lower cell concentrations (A_{540nm} = 0.25 to 2.00, Fig. 2) made it possible to calculate the percentage of the glass surface area covered by bacteria per unit of absorbance at 540 nm. After 40 min of incubation, an average of 11 bacteria adhered per 200 μm of the glass surface. Microscopic examination showed that bacteria sorbed singly or in small chains with all bacteria in close apposition to the glass. Supposing an average bacterial diameter of 1 μm, the percentage of area covered by bacteria was 4.3 per cent unit A_{540nm} after incubation for 40 minutes.

Effect of temperature. Increasing temperatures of incubation were accompanied by an increase in the number of bacteria sorbed (Fig. 4).

Effect of cell viability. Freshly prepared suspensions of bacteria in PBS were subjected to 100 °C for 10 min or to the radiation from a high-pressure mercury lamp (Osram HBO 200, *Osram GmbH, Berlin, Germany*) for 60 min. Both treatments resulted in more than 99.9 per cent killing of bacteria as related to the $1 \cdot 10^{9}$ colony forming units per ml of the untreated control suspension. Sorption was registrered after incubation for 0, 40 and 80 minutes. No significant effect on sorption of killing by radiation or of killing

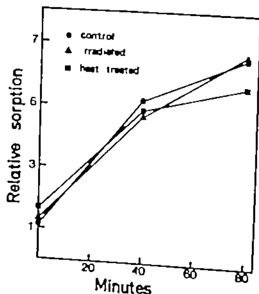


Fig. 5 Sorption to glass of heat-killed and UV irradiated cells of *S. faecium*. Means of 3 measurements.

RESULTS

Methodology Tritium labelled cells were assayed for cell integrity and retention of the labelled compound suspensions of labelled cells were repeatedly washed (three times each time) and resuspended (to the original volume) over a period of 21 h The absorbance at 540nm was read and scintillation counts were made after each washing and resuspension and the specific activity of the bacteria was calculated as counts per min (cpm) per unit A_{540nm} The results (Fig 1) showed that there was no detectable loss of label from intact organisms. However as an effect of storage or of the multiple washings, a decrease with time in the total number of cells was observed (Fig 1) It was concluded that in short term experiments scintillation counts were satisfactory as an estimate of bacterial cell number

The effect of sorption of bacteria to glass on counting efficiency was evaluated by comparing counts from 50 µl of suspension dried onto cover slips with counts from 50 µl added directly to the vial. Recovery of activity from bacteria dried onto glass relative to the suspended samples ranged from 101 to 107 per cent in four separate experiments.

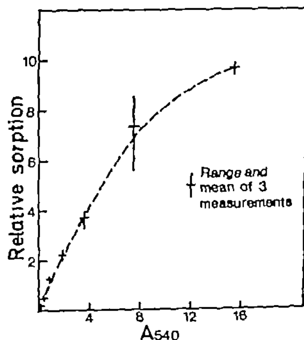


Fig 2 Sorption of *S faecium* to glass as a function of bacterial cell concentration. Incubation for sorption 40 min.

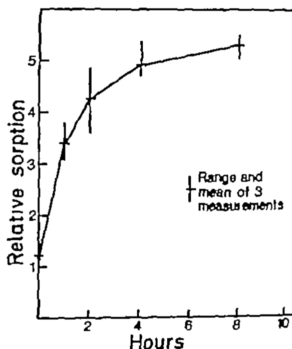


Fig 3 Sorption of *S faecium* to glass as a function of time

The standard curve obtained from 10 20, and 50 µl samples dried onto cover glasses was linear and experimental counts did not exceed the highest value on the standard curve.

Even after extensive washing small amounts of radioactivity were present in the supernatant. To investigate the possible adsorption of free thymidine or its derivatives to the glass cover glasses were incubated with the last washing liquid for 40 min No radioactivity was detectable on glasses thus treated, indicating that adsorption of radioactive material to the cover glasses would not interfere with measurements of bacterial sorption.

The glass-to-glass variation in scintillation count was estimated in a series of 9 triplets of identically treated samples. The extremes read 82 per cent and 121 per cent of the overall mean Average standard deviation within triplets was 7.33 per cent.

Effect on bacterial sorption of cell concen

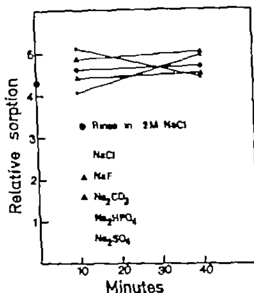


Fig. 8 Attempted desorption of glass-sorbed cells of *S. faecium* by various salt solutions. Incubation for sorption 65 min. Means of 3 measurements.

TABLE 1 *Desorption of Glass-sorbed S. faecium by NaCl, Urea, or Tween 80*

Incubation for sorption (min)	Bacteria remaining after 3 hours desorption (per cent of PBS controls)		
	2 M NaCl	4 M urea	2 % Tween 80
0	103	104	52
160	104	110	81

Suspensions of *S. faecium* in PBS were incubated with cover glasses for 5 (~0 min) or for 160 min. Control samples were then rinsed and immediately processed for counting of sorbed cells. Test samples were incubated for 3 h in the liquids for desorption; they were then rinsed and processed for counting. The test liquids were buffered at pH 7.4 with 0.01 M sodium phosphate buffer.

sorbed bacteria were incubated for three hours at room temperature in the fluids for desorption. Compared with the buffer control, urea and sodium chloride were ineffective as desorbing agents under these conditions (Table 1). Tween 80 on the other hand desorbed 48 per cent of the bacteria in the series incubated for 0 minutes and 19 per cent of those in the series incubated for 160 minutes (Table 1). In a separate experiment, 6 M urea buffered at pH 3.0 with glycine/HCl was also shown to be ineffective as a desorbing agent.

The desorbing effect of multiple washings with buffer was evaluated. After incubation for 0, 40, and 160 minutes, cover glasses with sorbed bacteria were rinsed once (3 s) respectively 10 times (30 s) with PBS. Mechanical detachment of bacteria was attained on a per cent basis more bacteria were susceptible to mechanical detachment after prolonged incubation (Table 2).

TABLE 2. *Mechanical Detachment of Glass-sorbed Cells of S. faecium*

Incubation for sorption (min)	Bacteria remaining after 10 rinses in PBS (per cent of controls rinsed once)
0	76
40	67
160	53

After incubation for sorption with PBS-suspended cells, cover glasses with sorbed bacteria were rinsed by dipping once (control) or 10 times (test) in PBS prior to counting of sorbed cells.

Finally desorption of bacteria was observed in the microscope (5). Ten µl of a suspension of bacteria was pipetted under a cover glass supported by two other cover glasses on a microscope slide. Sorption to and desorption from the cover glass was followed for one hour by phase contrast microscopy. Rapid sorption of bacteria was seen in the first few seconds of contact of the suspension with the glass. Unattached bacteria, however, sedimented rapidly from the surface and out of focus; continued sorption was therefore not possible. Sorbed bacteria were easily identifiable by the absence of Brownian motion. During the one-hour-observation period, only occasional organisms could be seen to detach from the cover glass.

Similar results were obtained if heat- or

and denaturation by heat could be observed (Fig 5)

Effect of salt concentration Solutions of KCl and NaCl were prepared at 10^{-1} 10^{-2} 10^{-3} and 10^{-4} M concentrations. Bacteria washed and redispersed in distilled water were added to each solution to a final density of A_{410nm} = 0 72 and sorption was recorded after incubation for 40 minutes A profound effect of the salt concentration was observed (Fig 6) very few bacteria sorbed at salt concentrations of 10^{-4} and 10^{-3} M but sorption increased dramatically through 10^{-2} to 10^{-1} M salt concentration

Effect of pH Suspensions of bacteria in saline were adjusted to pH ranging from 3 to 10.5 with citrate (3 to 6) phosphate (6.5 to 8) and glycine/NaOH (8.5 to 10.5) at a concentration of buffering substance of approximately 0 01 M Sorbed bacteria were counted after incubation for 40 minutes the results are shown in Fig 7 Sorption was maximal at a pH of 6.5 to 7 0 At pH levels above 7 0 sorption was noticeable less wehereas the lowering of pH below 6.0 only slightly reduced sorption.

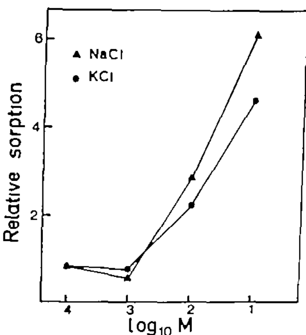


Fig 6 Sorption of *S faecium* to glass as a function of solute molarity Incubation for 40 min Means of 3 measurements.

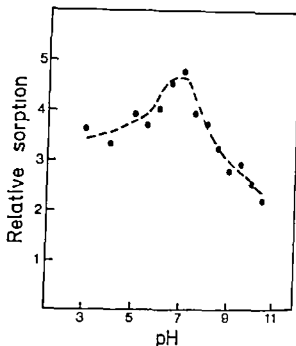


Fig 7 Sorption of *S faecium* to glass as a function of pH Incubation for 40 min. Means of 3 measurements.

Desorption of bacteria After incubation for 65 minutes in a bacterial suspension in 0 2 M NaCl (pH 6.5) cover glasses were transferred to 0.2 M of Na_2HPO_4 Na_2SO_4, Na_2CO_3 NaF or NaCl and left for 10 or 40 min Irrespective of the solutions tested any registrable desorption could not be observed (Fig 8)

It was also attempted to have bacteria desorbed from cover glasses by incubation in suspensions of unlabelled bacteria. After varying periods of desorbing time (0 10 and 40 min) no decrease in radioactivity measurements could be observed. Pre-incubation times required for sorption were 0 40 and 160 min.

Attempted desorption in distilled water likewise failed to detach bacteria already sorbed

The desorbing effect of 4 M urea 2 per cent Tween 80 and 2 M NaCl was tested The reagents were buffered at pH 7 4 by 0 01 M phosphate buffer Cover glasses were incubated with labelled bacteria in two series incubation for 0 and for 160 minutes. Following a brief rinse in PBS the glasses with

has been transceded by chemisorption. Physical adsorption is generally favoured by lower temperatures, whereas chemisorption proceeds at a higher rate at increasing temperatures. Also, equilibrium is usually reached very quickly in the case of physical sorption, and more slowly when chemisorption takes place. It appears that, in the sorption of *Streptococcus faecium* equilibrium is indeed not reached even after incubation for 8 hours. It seems unlikely that the concentration of bacteria (1 to 5 10^8 organisms per ml) was so low as to be rate limiting for a physical sorption process to reach equilibrium rather the formation of chemical bonds would require time and/or higher temperatures and be a more likely explanation of the continued sorption throughout several hours. The finding that sorption was favoured by higher temperatures further suggested that chemisorption was taking place.

In spite of the very high bacterial densities employed in this study there was a rather low area coverage (4.5 per cent per unit $A_{...}$ after 40 minutes) of the glass surface. A similar comparison between the concentration in suspension and the number sorbed has not been found in the literature however the area coverage is of the same order as, though generally higher than, that reported by *Bott & Brock* (1) *Marshall et al* (3) and *Meadows* (4) The low area coverage would seem to indicate that the soda-lime glass is highly inhomogeneous with respect to its affinity for the bacteria. Supposing that chemical bonds are formed between the glass and surface components of bacteria, only a very few "sites" on the glass surface present themselves as attractive for bacteria that are close enough to "recognize" the detailed surface chemistry of the glass (9) Such sites would probably vary with regard to their affinity for the bacteria, which would explain why the initial, quick rate of sorption rapidly and steadily decreases rapid saturation of high-affinity sites would be followed by slower sorption to the less "attractive" areas on the glass. That low affinity areas on the glass surface were involved at later stages was indicated by the greater percentage of bacteria which could be mechanically removed after longer incubation times.

The bonds formed between glass and bacteria would seem to be dissociated from any form of metabolic activities of the bacteria Nutritional substrates and energy were absent from the suspensions also sorption was unimpaired of bacteria that had been killed by heat or irradiation. An attempt was made to elucidate the nature of the bonds through desorption experiments with NaCl (ion exchange) urea (hydrogen bonds/hydrophobic interactions) and the non-ionic detergent Tween 80 (hydrophobic interactions) Effective desorption was accomplished only with Tween 80 implying that hydrophobic interactions are important in the chemisorptive process. This concept is supported by the potency of Tween 80 also in sorption inhibition. Consistent with this view is also the finding that cover glasses prehydrated for 24 h in buffer exhibit impaired qualities as substrate for bacterial sorption (unpublished observation)

Of the physico-chemical factors that may modify bacterial sorption and which have been investigated here only ionic strength appeared to exert any critical influence. Temperature and pH did show some, but in comparison minor effects on sorption. It seems safe to conclude that the ionic strength of the medium determines how close *S faecium* can come to the glass surface. Provided conditions thus are made favourable for apposition chemisorption of *S faecium* involving hydrophobic interactions may take place.

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formalin-killed cells were used in the desorption tests.

Interference with sorption Suspensions of labelled bacteria were incubated with cover glasses in the presence of NaCl (1 M) urea (2 M) and Tween 80 (1 per cent) Compared with the control suspension in PBS short time sorption (0 min incubation) was slightly inhibited in the presence of salt (16 per cent) and urea (16 per cent) and inhibited to a somewhat higher degree (35 per cent) by Tween 80 Inhibition of sorption by Tween 80 was more pronounced after incubation for 40 minutes (74 per cent) the effect of urea was also greater (30 per cent) whereas sorption in the presence of 1 M NaCl hardly differed from the buffer control (Table 3)

Similar results were obtained if heat or formalin killed bacteria were used

TABLE 3 *Interference with Sorption to Glass of S faecium by NaCl Urea or Tween 80*

Incubation for sorption (min)	Sorbed bacteria (per cent of controls suspended in PBS)		
	1 M NaCl	2 M urea	2 % Tween 80
0	84	84	65
40	93	70	26

Cells of *S faecium* were suspended in the test liquids (buffered at pH 7 4 with 0 01 M sodium phosphate buffer) or in PBS and incubated for sorption to cover glasses for 3 s ("0 min) or 40 min. The glasses were processed for counting of sorbed cells after one rinse in PBS

DISCUSSION

In the present investigation of bacterial sorption to glass, the influence of adsorbed nutrients (10) was minimized through the use of washed bacterial suspensions in buffer The absence of extraneous sources of energy or substrates for polymer production would furthermore limit, probably abolish, attachment mediated by polymers produced by the bacteria during incubation (5) Remaining for consideration would be the purely physical attraction between the glass and the bacterial cell and, possibly the establishment of chemical bonds directly from the glass to the bacteria This assumption was borne out by the fact that the results obtained with killed and live bacteria were essentially similar

Marshall et al (3) considered bacterial sorption to glass to entail an initial reversible phase of sorption. This initial sorption was shown to increase with ionic strengths up to 10^{-1} M of NaCl and $MgSO_4$ Their findings were compatible with the concept that the thickness of the electrical double layer which surrounds the bacteria and coats the glass, to a large extent determines the distance between the surfaces and the physical attraction between them. Results of the present investigation confirm the importance of the solute molarity It appears that the thickness of the double layer determines how closely an organism in suspension may approach the surface. However a number of observations in this study indicate that bacteria which sorb are not reversibly bound, but become irreversibly sorbed to the glass surface First bacteria could not be desorbed by a variety of salt solutions or by distilled water Second, incubation with unlabelled organisms did not either effect desorption Third prolonged incubation with 4 M urea or 2 M NaCl only slightly reduced the number of organisms sorbed to the glass Finally microscopic monitoring of bacterial sorption to glass indicated that sorbed bacteria which had lost Brownian motion did not detach These results are somewhat at variance with earlier reports (3 4) The differences may be dependent on the organism used for study the nonmotile *S faecium* strain may differ in sorptive characteristics from the motile species previously studied

Irreversible sorption implies the establishment of chemical bonds between the surface and the sorbed particle Electrical parameters, such as the zeta potential and the electrical double layer are then insufficient to explain the association physical adsorption

Acta path. microbiol. scand. Sect. B, 85: 47–55, 1977

SORPTION OF STREPTOCOCCI TO GLASS: EFFECTS OF MACROMOLECULAR SOLUTES

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Ørstavik, D. Sorption of streptococci to glass: effects of macromolecular solutes. Acta path. microbiol. scand. Sect. B, 85: 47–55, 1977.

Macromolecular solutes (albumin, concanavalin A, whole saliva, serum) caused impaired sorption of *Streptococcus faecium* and *Streptococcus sanguis* to glass. The inhibitory effect resided chiefly with interactions of the solutes with the glass surface. In the case of sorption of *S. sanguis* to glass in the presence of parotid fluid, the inhibitory effect was counteracted by specific attachment of *S. sanguis* cells mediated by some component(s) of the parotid fluid. Agglutination of the test organisms was in general accompanied by inhibition of sorption. However, when small or unstable aggregates were formed, the number of cells adhering on the glass surface was increased. The findings are discussed with reference to the colonization of teeth by oral bacteria.

Key words: Sorption of streptococci; glass; macromolecular solutes.

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Received 8.viii.76 Accepted 10.ix.76

Bacterial sorption to solid surfaces may occur through direct interactions between the bacterial and the solid surfaces (13, 15). In natural environments, however, such interactions are more often than not modulated by macromolecular solutes. In the mouth, for instance, the colonization of teeth by oral bacteria is preceded by the formation of an acquired pellicle on the enamel surface (1). This pellicle, which is formed by organic solutes present in saliva (14), promotes the *in vitro* adherence of certain streptococci to the tooth surface (15). On the other hand, salivary immunoglobulins (17) and glycoproteins (18) may inhibit the *in vitro* adherence of oral streptococci to epithelial cells by direct interaction with the bacterial surface. In the latter case, adherence inhibition is accompanied by agglutination of the test organism.

In a previous study (13), a model system for bacterial sorption to glass *in vitro* was described. This model system has been applied in the present experiments to evaluate the effects of macromolecular biological solutes on the sorption of two streptococcal strains to glass.

MATERIALS AND METHODS

Bacteria. A laboratory strain of *Streptococcus faecium* (13) and one streptococcal strain isolated from human dental plaque, were used. The latter strain, designated OI 5, was tentatively considered to belong to Carlsson's (3) *Streptococcus sanguis* group I A by the following criteria: it gave alpha-haemolysis on blood agar; its colonies adhered to mitis-salivarius agar; it produced hydrogen perox

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TABLE 1 *Influence of Macromolecular Solutes on the Sorption to Glass of* Streptococcus faecium *and* Streptococcus sanguis

Medium	*S faecium* I	II	III	*S sanguis* I	II	III
HSA 2.5 mg/ml	17	18	93	16	37	111
OVA 2.5 mg/ml	19	20	81	50	64	84
Con A 2.5 mg/ml	44	28	64	36	25	82
RS 2 3 in PBS	14	24	65	16	52	–
Whole saliv	27	18	88	37	51	108
Parotid fluid	52	29	59	78	90	119
PF absorbed	–	–	–	–	22	–
PBS	100	100	100	100	100	100

Not done. HSA: human serum albumin OVA: egg albumin Con A concanavalin A (*Pharmacia*) RS rabbit serum PF absorbed parotid fluid absorbed (1 h 37 C, overnight 4 C) with washed sediments of *S. sanguis*. Figures represent sorbed cells in per cent of PBS controls.

I series I the medium was present during incubation for sorption, which was for 40 min at room temperature. I series II the cover glasses had been pretreated by incubation with the media for 3 h at room temperature followed by one rinse in PBS prior to their incubation for sorption with suspensions of the bacteria in PBS. In series III the bacteria had been pretreated by incubation with the media for 1 h at 37 C followed by one wash and resuspension in PBS prior to their incubation for sorption to clean cover glasses.

same subject from whom *S sanguis* O15 was isolated.

EXPERIMENTS AND RESULTS

Bacterial sorption in the presence of organic solutes. Bacteria were suspended in protein solutions, in oral fluids, or in dilute rabbit serum during incubation for sorption to cover glasses. The details of the experimental conditions are given in the legend to Table 1 The presence of organic solutes reduced the sorption of *S faecium* to glass by some 50 to 85 per cent. In general, reduced sorption was observed also if *S sanguis* were used how ever this strain sorbed to cover glasses at a level approaching the PBS control (78 per cent) when parotid fluid was used as suspension medium (Table 1) *S faecium* was tested for sorption also after agglutination by varying concentrations of Con A, and sorption inhibition was related to the degree of agglutination caused by Con A. The results (Fig 2) showed that both inhibition of sorption and agglutination increased with increasing concentrations of Con A, and the dose dependence of the two parameters was quite similar

Bacterial sorption to pretreated cover glasses Details of these experiments are described in the legend to Table 1 When cover glasses had been pre incubated with protein solutions or body fluids, the reduction in bacterial sorption relative to sorption to PBS-preincubated glasses would generally be marked. The effect was most notable in the case of *S faecium* whereas sorption of *S sanguis* to glasses pre treated with parotid fluid was almost unimpaired, and less inhibited than sorption of *S faecium* in most other tests. The exceptionally high levels of *S sanguis* sorbing to glasses pretreated with parotid fluid prompted a separate experiment. Parotid fluid was absorbed by incubation (1 h 37 C, overnight 4 C) with washed cells of *S sanguis* and centrifuged before incubation with cover glasses. Sorption of *S sanguis* to these glasses was dramatically reduced as compared with both PBS and unabsorbed parotid fluid controls (Table 1)

Sorption of bacteria pretreated with organic solutes. Bacteria were incubated for 1 h at

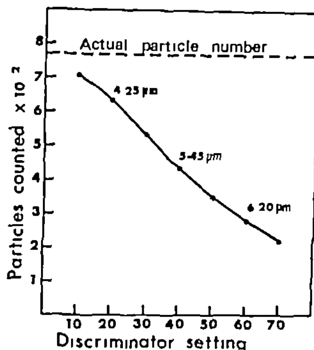


Fig 1 Calibration of electronic cell counter for agglutination measurements. A suspension of latex particles was counted in the haemocytometer and a suitable dilution for counting in the cell counter was prepared The diluted suspension was counted at various discriminator settings as shown, while the current was set constant at 20 Diameter exclusion limits, based on calculations assuming a normal size distribution of the spheres, are indicated

ide it did not ferment ribose trehalose sorbitol or mannitol it produced ethanol precipitable polysaccharides in 5 per cent sucrose broth it did not produce ammonia from arginine However the strain did not exhibit spreading on blood agar plates after prolonged incubation in a humid atmosphere (8) nor did it react with Lancefield group H antiserum or with an antiserum to a recently discovered, separate antigen of *S sanguis* (9) Cultural conditions and procedures for the standardization of bacterial suspension in phosphate-buffered saline (PBS) have been described (13)

Bacterial sorption to glass. Details of the procedure for measuring bacterial sorption to glass have been described (13) Briefly test suspensions of bacteria labelled with H thymidine were incubated with microscope cover glasses at room temperature for varying periods of time The glasses were subsequently washed for 5 s in buffer air-dried transferred to liquid scintillation vials, and treated with formamide for 2 h at 37 C to dissolve bacterial cell material After the addition of scintillation liquid the vials were placed in a Packard Tri-Carb 2450 liquid scintillation counter All samples were prepared and counted in triplicates the means of which have been entered in the results. Individual measurements deviated on the average by 7.3 per cent (range 0–21 per cent, n = 27) from the triplet mean value.

Measurement of bacterial agglutination. An electronic cell counter (Celloscope 101 *AB L. Ljungberg & Co Stockholm*) was used to quantitate bacterial agglutination (11) The instrument's capillary cell had a 100-µm diameter orifice and 1 00 ml of sample passed the orifice in approximately 31 s. For calibration of the instrument, a test suspension of latex particles was used. According to the manufacturer (*Serva Heidelberg, W Germany*) the latex spheres had a mean diameter of 5 7 µm with a standard deviation of 1.50 µm The actual number of latex particles was counted in a haemocytometer and a suitable dilution for counting in the cell counter was made. This suspension was then counted at a constant current (setting at "20" on the current regulator) but at different settings on the aperture discriminator The resulting counts are shown graphically in Fig 1 Supposing a normal size distribution of the latex spheres, an exclusion limit below which particles are not counted can be calculated for each discriminator setting. A discriminator setting of 20 corresponding to an exclusion limit of 4.25 µm, was chosen for subsequent work with bacterial aggregates, as most aggregates of saliva-agglutinated streptococci would be expected to have a diameter greater than 4.25 µm (11) For agglutination tests, suspensions of the streptococci in PBS (optical density at 540 nm = 1 0–2.0) were incubated with the agglutinating agent for 1 h at 37 C and overnight at 4 C. The samples were shaken for 15 s on a Vortex mixer and 0.5 ml of the sample was transferred to 15 ml PBS and counted electronically Absolute counts of bacterial aggregates were not calculated in these experiments relative agglutination only was registered within each test series. All counts were done in triplicates, the means of which have been entered in the results. Individual measurements deviated on the average by 6 4 per cent (range 0–22 per cent n = 30) from the triplet mean value

Reagents Human serum albumin, egg albumin and concanavalin A (Con A, grade I S) were obtained from *Sigma*. A preparation of Con A was also obtained from *Pharmacia*. Rabbit serum was pooled from 4 untreated animals. Parotid fluid from one subject was stimulated by lemon candy and collected through a Curby cap (4) in ice-chilled test tubes. Paraffin-stimulated whole saliva from the same subject was likewise collected in ice-chilled tubes it was immediately centrifuged (10 000 × g 4 C 10 min) to remove particulate matter The parotid fluid and the whole saliva were stored frozen at −90 C prior to use The donor of parotid fluid and whole saliva was the

TABLE 2. *Sorption to Glass of Agglutinated Bacteria*

Agglutination system	Suspension tested	Sorption	Agglutination
S faecium concanavalin A	unwashed	16	200
	washed	41	160
	washed and disrupted	81	17
	non-agglutinated control	100	5
S sanguis whole saliva	unwashed	45	2+
	washed	116	1+
	washed and disrupted	152	0
	non-agglutinated control	100	0

Figures applying to sorption represent sorbed cells in per cent of PBS controls. Agglutination of *S faecium* was read electronically agglutination of *S sanguis* was graded (0–4+) by microscopic observation.

S faecium was agglutinated by incubation in 0.1 mg/ml Con A (*Pharmacia*) for 1 h at 37 C and over night at 4 C. *S sanguis* was agglutinated by similar incubation in whole saliva 1:2 in PBS Cells incubated with PBS only served as controls. The agglutinated cells were suspended by shaking on a Vortex mixer for 15 ; they were then divided into three equal parts. One part (unwashed) was tested for sorption (40 min, room temperature) and agglutination directly Another part (washed) was washed 3 times and resuspended in PBS prior to sorption and agglutination tests. The third part (washed and disrupted) was washed, resuspended, and disrupted by treatment for 10 s in a MSE 100 W ultrasonic disintegrator (*Measuring and Scientific Equipment Ltd., London*) operating at 20 kH and with 6 μm amplitude.

TABLE 3. *Sorption to Glass of S* faecium *Agglutinated by Tris®/Ca⁺⁺*

$CaCl_2$ (mM)	pH 9.2		pH 11.8		Sorption at pH 11.8 corrected for Ca^{++}-dependency
	Sorption	Agglutination	Sorption	Agglutination	
0	100	5	100	5	
.2	116	6	186	5	160
1	121	5	173	8	143
2	150	4	122	10	81
20	158	5	102	334	65

Sorption is expressed as per cent of sorption in Ca^{++}-free Tris® buffer The figures in the right hand column are sorption percentages at pH 11.8 related to sorption percentages at pH 9.2. Agglutination was measured electronically

Cells of *S faecium* were suspended in 0.1 M Tris® containing concentrations of $CaCl_2$ as indicated. pH was adjusted to 9.2 and 11.8 by the addition of 0 1 N NaOH. Incubation for agglutination covered 30 min at room temperature. The samples were then shaken for 15 s on a Vortex mixer prior to testing for sorption (40 min, room temperature) and agglutination.

tion was increased up to 60 per cent relative to the pH 9.2 control.

DISCUSSION

In a previous publication (13) the sorption of *S faecium* to glass was shown to be an essentially irreversible process probably dependent on the formation of chemical bonds between the bacterial and glass surfaces. As demonstrated in the present study sorption of *S faecium* to glass will generally be markedly reduced in the presence of organic solutes thereby indicating that macromolecular solutes may interfere with this bonding. Such an effect might have been brought about

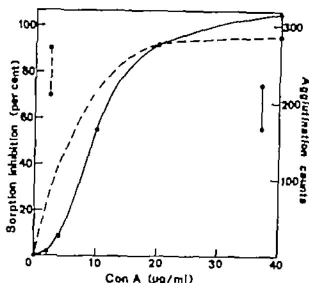


Fig 2 Sorption to glass of *Streptococcus faecium* agglutinated by concanavalin A. *S faecium* was incubated in PBS containing 0 2 4 10 20 and 40 µg/ml of Con A (*Sigma*) for 1 h at 37 C and overnight at 4 C. The samples were suspended by shaking on a Vortex mixer for 15 s prior to incubation for sorption, which was for 40 min at room temperature.

37° C with the various solutes as described in the legend to Table 1 Agglutination could not be registered either visually or electronically after this treatment. The bacteria were tested for sorption to clean cover glasses following centrifugation one wash and resuspension in PBS Compared with sorption in the presence of the solutes sorption of pretreated bacteria was increased and approached the level of sorption in the absence of organic solutes (Table 1) Indeed, sorption of *S sanguis* thus treated tended to exceed the PBS control value (Table 1)

Effect of agglutination on bacterial sorption The results of the experiments described in Fig 2 indicated a relationship between agglutination and inhibition of sorption. To evaluate the effect of agglutination *per se* cells of *S faecium* agglutinated by Con A were tested for sorption to glass after 3 times wash and resuspension in PBS Besides, the effect of ultrasonic disruption of the aggregates was tested. The results (Table 2) showed that removal of unbound Con A by washing caused some 25 per cent increase in sorption relative to sorption of agglutinated cells in the presence of free Con A. However the washing procedure was accompanied by a reduction in the number of aggregates, a reduction of a magnitude (20 per cent) similar to that of the increase in sorption. Similarly when washed aggregates were disrupted by ultrasonic treatment the increase in sorption (80 per cent of PBS controls) was accompanied by a comparable decrease in the number of aggregates (92 per cent)

A similar experiment was performed with *S sanguis* agglutinated by whole saliva. These aggregates appeared unstable and were not suitable for electronic counting Sorption inhibition was therefore related to an index of agglutination (0-4+) based on microscopic observation of the samples after their suspension on the Vortex mixer Inhibition of sorption was noted only when whole saliva was present during incubation for sorption (Table 2) Washing of the saliva-agglutinated *S sanguis* cells resulted in a decrease in the agglutination index from 2+ to 1+ but whereas some aggregates still persisted after this treatment, sorption exceeded the PBS control level by 16 per cent. Complete dispersal of these aggregates by ultrasonic treatment further increased sorption to some 150 per cent of the PBS control level

To evaluate the effect on sorption of non polymer-dependent agglutination a system involving *S faecium* 0 1 M Tris® (*Sigma*) and varying concentrations of $CaCl_2$ was employed. At 20 mM Ca^{++} and at a pH of appr 11.5 cells of *S faecium* agglutinated within 30 min in this system. These agglutinates were unstable and dissociated when washed in PBS or in Ca^{++} free Tris solutions. Therefore, to separate the effect of agglutination from the effect of the solute *per se* sorption of Ca^{++}/pH-aggregated cells was related to sorption in the presence of identical concentrations of Ca^{++} but at a lower pH where agglutination did not occur (Table 3) The results showed that at higher concentrations of Ca^{++} agglutination was accompanied by sorption inhibition (Table 3) However at subaggregating concentrations of Ca^{++} sorp-

pellicle *in vitro* (15) and by the present observation of high levels of *S. sanguis* adhering to cover glasses treated with parotid fluid.

The variable influence of whole saliva as a suspension medium on the *in vitro* attachment of *S. sanguis* to the acquired pellicle (15) may also be interpreted in the light of the present findings. Sorption and subsequent attachment of bacteria may be promoted if the degree of agglutination is low, whereas it will be reduced if the degree of agglutination is high. Indeed, agglutination of *S. sanguis* suspended in parotid fluid is pronounced, and the same is true of inhibition of attachment to the acquired pellicle, whereas whole saliva causes little agglutination and its influence on attachment is variable (15).

The nature of the determinants responsible for bacterial attachment to the acquired pellicle remains to be elucidated. However, they must combine specificity for bacteria with affinity for the enamel surface or for other pellicle constituents. Salivary substances capable of agglutinating oral bacteria show variable, but generally high, affinity for hydroxyapatite (6, 7) and such agglutinating substances may promote bacterial attachment when incorporated in the acquired pellicle.

Dr *S. D. Henriksen* kindly performed the antigenic analyses and the test for bacterial spreading. The help of Dr *K. Helgeland* and Dr *P. Brandtzaeg* in reviewing the manuscript is appreciated, as is the technical assistance of Ms. *Eleftheria Sterrag*. The study was supported in part by the *Norwegian Research Council for Science and the Humanities*.

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by interactions of the solute with the bacteria, with the glass or with both. Since sorption of bacteria pre incubated with the solutes hardly was affected, whereas sorption was inhibited when the cover glasses had been pre incubated, it may be concluded that the inhibitory action of the macromolecular solutes resides chiefly with their interactions with the glass surface. During work with the adsorption of dextran-coated *S mutans* cells to hydroxyapatite, *McGaughey et al.* (12) observed also that saliva inhibited sorption through its reaction with the apatite not with the bacterial surface.

Assuming that bacteria sorb to affinity "sites on the glass surface (13) inhibition of sorption by solute macromolecules may be accomplished through their interactions with these sites. Albumin preparations, as well as human saliva, form surface films on glass surfaces (2, 16) Film formation will modulate the critical surface tension which probably is one important determinant of sorption (5) However the surface film may also introduce lectin like, bacteria-specific determinants that may bind selected bacterial species. Such binding may more aptly be termed *attachment* rather than *sorption*, as it is mediated by extracellular polymers spanning the gap from the bacteria to the solid surface. The high levels of *S sanguis* on glass pretreated with parotid fluid may be explained by an attachment of this kind. While pre incubation with parotid fluid in general may impair the sorptive qualities of glass (cf experiments with *S faecium*) substances in parotid fluid with high affinity for *S sanguis* may promote attachment when incorporated in the glass surface film Separate experiments (data not shown) demonstrated that *S sanguis* was agglutinated by parotid fluid, whereas *S faecium* remained unaffected. The dramatically reduced sorption of *S sanguis* to glasses pretreated with absorbed parotid fluid may thus be explained by assuming that the factor(s) responsible for attachment had been removed.

Solutes non reactive with the bacteria inhibited sorption mainly through surface film formation In the case of solutes that caused agglutination of the test organisms, it was more difficult to distinguish between the effect on sorption of film formation and of agglutination. On the one hand Con A agglutinated cells of *S faecium* exhibited impaired sorption also after removal of free Con A. On the other hand sorption of saliva suspended *S sanguis* was inhibited even though the time of incubation was too short for agglutination to be accomplished Solutes which cause agglutination of the bacteria may thus inhibit sorption both by surface film formation and by agglutination. Another possible mechanism *viz.* cell surface modification by the agglutinating substance, was not investigated here. The relationship between agglutination and sorption, however may be still more complex washed cells of saliva treated *S sanguis* and cells of *S faecium* at subaggregating concentrations of Ca^{++} exhibited increased sorption relative to controls. It may be that these small bacterial aggregates, in contrast to the large aggregates formed by Con A and at higher Ca^{++} concentrations, readily sorb to the glass surface Thus, whereas sorption is impaired by the formation of large aggregates, small aggregates may sorb *en bloc* thereby increasing the number of organisms on the glass surface

The findings in the present study may have a bearing on the events occurring during early dental plaque formation. Sorption and attachment of oral bacteria to the tooth surface initiates plaque accumulation and the attachment of certain streptococci is mediated by an adsorbed acquired pellicle on the enamel surface (15) Other *in vitro* experiments have shown a variable influence of saliva on the sorption of bacteria to powdered enamel (10) Extrapolation from the present findings indicates that the formation of an acquired pellicle generally will render the enamel surface less suitable as substrate for bacterial sorption However specific chemical groups of the pellicle may act as determinants for the attachment of selected oral bacteria. This concept is supported by the finding of selective attachment of *S sanguis* to the acquired

MATERIALS AND METHODS

Chemicals Chrystallised trypsin from bovine pancreas was obtained from E. Merck A.G., Darmstadt, Germany. It contained 2 proteolytic units per mg. Pronase from *Streptomyces griseus* containing 45 proteolytic units per mg was purchased from Koch-Light Laboratories, England.

Cells The origin of the human embryo lung (HEL) cells, HeLa cells and L-F_1 mouse fibroblasts employed in this study has been reported (9). They were all grown in a mixture of Eagle's medium and medium 199 (50:50) supplemented with 10 per cent inactivated calf serum and 0.044 per cent $NaHCO_3$. In all tests for antiviral and cell growth inhibitory activities the same medium with 2 per cent serum and 0.088 per cent $NaHCO_3$ was used.

Viruses Vesicular stomatitis virus (VSV) Indiana strain, was used as challenge virus in tests for antiviral activity. The Sendai strain of parainfluenza 1 virus was employed as inducer of IF production in human leukocytes. Both viruses were produced in the allantoic cavity of embryonated hens eggs.

Interferon Sendai virus induced human leukocyte IF was produced by the method of *Cantell et al* (6). In some experiments concentrated preparation of leukocyte IF was used. This preparation was received as a gift from Dr. *K. Cantell*, Helsinki.

Preparation of control interferon A suspension of freshly harvested human leukocytes was divided in two equal volumes. One was used for production of lekocyte IF the other for control IF. The two suspensions were treated in the same way except that the control suspension was not induced with Sendai virus. Interferon and control preparations were stored at −20° C in small volumes.

Test for antiviral activity The infectivity inhibition micro method has been described in detail elsewhere (7, 9). A laboratory standard preparation of human leukocyte IF was included in each test. All titres are corrected to the International standard of human IF 69/19 B, and expressed as $\log_{10}$ reference units per 0.1 ml.

Test for cell growth inhibitory activity The test for cell growth inhibitory activity has been described (9). In short tubes were seeded with 10^5 cells in 1 ml of medium containing the desired dilution of test material. At least 5 parallel tubes were used. Control tubes were seeded in medium containing the appropriate buffer in the same dilution. After 3 days of incubation at 37° C in 5 per cent CO_2 atmosphere the cultures were treated with trypsin-versene and counted in haemocytometer. In some representative cases, the counting was performed after vital staining with Trypan Blue. No difference in the counts with and without staining was observed. If not otherwise stated in the text, HEL cells were used in all tests for cell growth inhibitory activity.

Preparation of purified growth inhibitory component Human lekocyte IF was chromatographed on albumin-agarose (8). Each fraction (5 ml) was tested for antiviral and cell growth inhibitory activities. The fractions eluted with 1 M NaCl containing maximal GIA and low titres of antiviral activity were pooled and concentrated by lyophilization. The material was purified by gel-filtration on Sephadex G-25 and stored at −20 C in small volumes.

RESULTS

Biological and Physico-Chemical Characterization of the Growth Inhibitory Factor of Unseperated Interferon

It has been suggested that the antiviral and growth inhibitory activities are caused by the same molecule (16). If so, the two effects may change in parallel by any treatment.

Human leukocyte IF was treated by methods usually employed to characterize IF and tested in HEL cells for GIA and antiviral activity. The results given in Table I show that the two activities did vary in parallel.

These results are in agreement with those reported by others (1, 16). They may suggest that the growth inhibitory effect is caused by a protein similar to—or identical with—the antiviral IF molecule itself. However after separation on albumin-agarose (8) all cell growth inhibitory activity was lost when fractions with strong GIA were dialysed against PBS. This indicates that a small molecules is responsible for the effect on cell growth. In agreement with this, the mol. wt. estimated by gel-filtration on Sephadex G-25 was approx. 3200 (8).

These results indicate that the GIA-component is a molecule distinct from IF. Therefore, the following experiments were concentrated on a characterization of the purified GIA-component in relation to the accepted properties of IF.

Acta path. microbiol. scand. Sect. B 85 54–60 1977

HUMAN INTERFERON AND CELL GROWTH INHIBITION

II Biological and Physico-Chemical Properties of the Growth Inhibitory Component

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Dahl H. Human interferon and cell growth inhibition. II Biological and physico-chemical properties of the Growth Inhibitory Component. Acta path. microbiol. scand. Sect. B, 85 54–60 1977

Human leukocyte interferon (IF) inhibits the growth rate of homologous cells in culture. The growth inhibitory component, formerly separated from the antiviral component of human leukocyte IF by adsorption chromatography on albumin-agarose, is investigated. The properties of the component are compared with the accepted characteristics of IF The growth inhibition is caused by a small molecule, unstable to pH 2 treatment with HCl, but stable to trichloro-acetic acid (TCA) and to proteolytic enzymes The isolated growth inhibitor seems to be a dimer of molecular weight (mol wt.)~2300. It is activated by heat treatment and lacks species specificity The discrepancies in the properties of the growth inhibitor before before and after separation from IF are discussed.

Key words Human interferon cell growth inhibition growth inhibitory component.

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Received 31 viii 76 Accepted 30.ix.76

Human interferon (IF) exhibits a growth inhibitory activity (GIA) on homologous cells (3 5 9 11 12 16 17) comparable to that of mouse IF preparations. This effect was strictly correlated to the antiviral titre in most experiments (8 9 13 14 22) independent of degree of purification The growth inhibitor has been suggested to be a protein indistinguishable from IF (16) However disproportionality between the two effects of mouse IF has also been reported (2 10 20 21)

Recently an apparently complete separation of the GIA from the antiviral activity of human leukocyte IF was reported from our laboratory (8) The separation was performed by adsorption chromatography on an albumin-agarose column Urea treatment of leukocyte IF followed by dialysis against 1 M NaCl resulted in the complete loss of GIA (8) The results suggested that the GIA of leukocyte IF is a distinct molecule attached to the IF protein by electrostatic bonds deep inside the IF molecule.

In the present paper some of the physico-chemical properties of the GIA-component are reported and compared with the accepted properties of IF

MATERIALS AND METHODS

Chemicals Chrystallized trypsin from bovine pancreas was obtained from E. Merck A.G Darmstadt, Germany It contained 2 proteolytic units per mg. Pronase from *Streptomyces griseus* containing 45 proteolytic units per mg was purchased from Koch-Light Laboratories, England.

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Preparation of control interferon A suspension of freshly harvested human leukocytes was divided in two equal volumes. One was used for production of lekocyte IF the other for control IF The two suspensions were treated in the same way except that the control suspension was not induced with Sendai virus Interferon and control preparations are stored at 20° C in small olumes

Test for antiviral activity The infectivity inhibition micro method has been described in detail elsewhere (7 9) A laboratory standard preparation of human leukocyte IF was included in each test. All titres are corrected to the international standard of human IF 69/19 B, and expressed as log reference units per 0.1 ml.

Test for cell growth inhibitory activity The test for cell growth inhibitory activity has been described (9) In short tubes were seeded with 10⁴ cells in 1 ml of medium containing the desired dilution of test material. At least 5 parallel tubes were used Control tubes were seeded in medium containing the appropriate buffer in the same dilution After 3 days of incubation at 37° C in 5 per cent CO_2 atmosphere the cultures were treated with trypsin-versene and counted in haemocytometer I some representative cases, the counting was performed after vital staining with Trypan Blue No difference in the counts with and without staining was observed. If not otherwise stated in the text, HEL cells were used in all tests for cell growth inhibitory activity

Preparation of purified growth inhibitory component Human lekocyte IF was chromatographed on albumin-agarose (8) Each fraction (5 ml) was tested for antiviral and cell growth inhibitory activities. The fraction eluted with 1 M NaCl containing maximal GIA and low titres of antiviral activity were pooled and concentrated by lyophilization. The material was purified by gel-filtration on Sephadex G-25 and stored at −20 C in small volumes.

RESULTS

Biological and Physico-Chemical Characterization of the Growth Inhibitory Factor of Unseparated Interferon

It has been suggested that the antiviral and growth inhibitory activities are caused by the same molecule (16) If so, the two effects may change in parallel by any treatment.

Human leukocyte IF was treated by methods usually employed to characterize IF and tested in HEL cells for GIA and antiviral activity The results given in Table 1 show that the two activities did vary in parallel.

These results are in agreement with those reported by others (1 16) They may suggest that the growth inhibitory effect is caused by a protein similar to—or identical with—the antiviral IF molecule itself However after separation on albumin-agarose (8) all cell growth inhibitory activity was lost when fractions with strong GIA were dialysed against PBS This indicates that a small molecules is responsible for the effect on cell growth. In agreement with this, the mol. wt. estimated by gel-filtration on Sephadex G-25 was approx. 5200 (8)

These results indicate that the GIA-component is a molecule distinct from IF There fore, the following experiments were concentrated on a characterization of the purified GIA-component in relation to the accepted properties of IF

TABLE 1 *Physico-Chemical Characterization of the Antiviral and the Growth Inhibitory Activities of Human Leukocyte Interferon*

Treatment	Log_2 units of IF per 0.1 ml	Per cent reduction in cell count*
Untreated IF	3.0	35.0
pH 2 for 2 days (HCl)	2.9	34.5
pH 2 for 2 days (TCA)	<1.0	0.0
0.5 mg trypsin per ml, 37 C, 1 hour	<1.0	0.0
37 C for 1 hour	3.0	34.8
56 C for 1 hour	2.7	24.5
Dialysis against PBS for 24 hours	2.9	35.5
Control IF	<1.0	0.0
Activity on mouse L-F_1 cells	1.2	14.5

* Samples were tested in dilution 1/100 (100 units of untreated IF per ml)

pH 2 Treatment

Samples of purified GIA-component were adjusted to pH 2 by either 1 N HCl or 40 per cent TCA and kept at 4° C As control a sample was kept at 4° C without pH 2 treatment. After 2 days the samples were neutralized with 1 N NaOH and tested for GIA on HEL cells. The results given in Table 2 show that the GIA-component is unstable to HCl, but stable to TCA treatment. The difference from the effect of pH 2 treatment on unseparated IF is striking

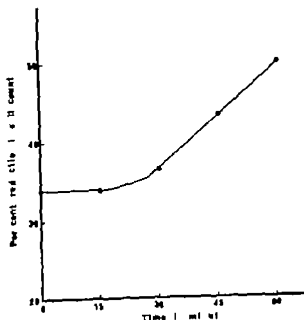


Fig 1 Effect of heat treatment on the activity of the GIA-component. Samples were incubated for the indicated periods of time at 37 C in a water bath then transferred to an ice bath before testing for effect on cell growth.

TABLE 2 *Physico-Chemical Characterization of the GIA-component*

Experiment number	Sample	Per cent reduction in cell count
I	pH 2 (HCl)	0.0
	pH 2 (TCA)	21.1
	Control	25.1
II	Untreated	34.1*
	0.5 mg trypsin per ml 37 C, 1 hour	46.5
	5 µg pronase per ml, 37 C, 1 hour	47.3
	37 C for 1 hour	46.1
	56 C for 1 hour	39.3

* Standard deviation 3.1

Proteolytic Digestion and Stability to Heat

Samples of purified GIA-component were incubated in a water bath at 37° C or 56° C for one hour with and without 0.5 mg trypsin or 5 µg pronase per ml as indicated in Table 2 The enzyme activities were stopped by addition of TCA to a final concentration of 10 per cent. The pH was adjusted to neutrality and the samples tested for effect on cell growth. The results are shown in Table 2

The GIA-component was not digested by trypsin or by the more unspecific proteolytic enzyme, pronase. Heating at 56 C enhanced the activity slightly while heating at 37 C resulted in a strong enhancement in activity (as the difference exceeded 3 standard deviations the enhancement in activity after 37 C is considered significant)

The observed activation of the GIA-component was further investigated. Samples of GIA-component were incubated in a water bath at 37 C for the indicated periods of time and tested for effect on cell growth. The results shown in Fig 1 confirm the observed activation. After one hour no further increase in activity was observed.

Estimation of Molecular Weight

In a preliminary experiment (6) the mol. wt. of the GIA-component has been estimated to approx. 3200 by gel-filtration on Sephadex G-25 The observed enhancement in activity by heating may suggest a dimeric or oligomeric arrangement of the molecule. To test this possibility a sample of purified GIA-component was incubated for one hour at 37 C and then applied to a Sephadex G-25 column (1.2 × 31 cm) The column was eluted with 0.15 M NaCl in 3 ml fractions. An untreated sample was gel-filtered in the same way All fractions were tested for effect on cell growth in dilution 1/20 The elution profiles from both runs are shown in Fig. 2. Gel-filtration of the untreated GIA-component gave rise to a single peak with mol. wt. approx. 2300 Incubation at 37 C resulted in the appearance of two additional peaks of mol. wt. approx. 4000 and 1100 respectively

Species Specificity Dose Response Curves

When tested in mouse L-F cells, the antiviral titre of human leukocyte IF was about 100 fold lower than in homologous cells and the GIA correspondingly low (Table 1) The purified GIA-component was tested for effect on the growth of HEL, HeLa and L-F cells in the indicated dilutions (Fig 3) L-F cells and HeLa cells were identically affected. As the two cell lines have similar growth rate,

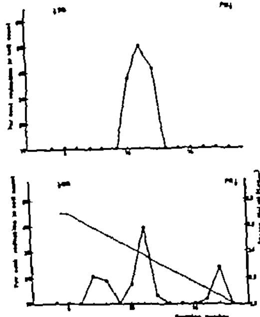


Fig. 2 A Gel-filtration on Sephadex G-25 of purified GIA-component. Fractions (3 ml) were eluted with 0.15 M NaCl and tested for effect on cell growth in dilution 1/20 B Gel-filtration on Sephadex G-25 of purified GIA-component after activation at 37° C for 1 hour. Fractions were eluted and tested as in A. BD Blue Dextran, high mol. wt. marker (>5000) PR Phenol red, low mol. wt. marker (<1000)

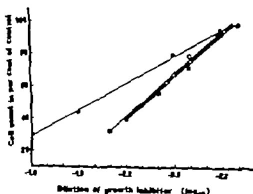


Fig 3 Dose-response curves for the effect of GIA-component on different cell lines. ● HEL, ○ HeLa, ■ L-F.

this result suggests that the GIA-component completely lacks species specificity. The more slowly growing HEL cells were less affected. This confirms earlier indications that the growth inhibition is somehow correlated with growth rate (9, 17).

DISCUSSION

Earlier reports suggest that the growth inhibitory activity of IF preparations is caused by IF itself or by a protein indistinguishable from IF (16). In contrast, former results from our laboratory show that a GIA-component can be separated from human leukocyte IF (8). According to the present findings this activity is due to a small molecule fundamentally different from IF. The GIA-component was unstable to pH 2 treatment with HCl, but stable to TCA treatment (Table 2, exp. I). It was not digested by trypsin—or by pronase—and was activated by heat (Table 2, exp. II). All activity was lost by dialysis against PBS (8) in agreement with the estimated mol. wt. of ~2300 (Fig. 2). In addition the GIA-component lacks species specificity as demonstrated in mouse L-F_1 cells (Fig. 3).

In contrast to IF, the GIA-component was activated by heat treatment. The simultaneous appearance of growth inhibitory factors with higher mol. wt. (~4000) and lower mol. wt. (~1100) (Fig. 2) may explain the increase in activity. The two additional peaks probably represent the tetramer and the monomer of the molecule respectively. Thus, the isolated purified GIA-component of mol. wt. ~2300 may represent the dimer consisting of identical monomers and with a tendency to polymerize. Formerly the mol. wt. was estimated to approx. 3200 (8). This estimate was based on larger Sephadex G 25 fractions (5 ml) and less purified material was used. In addition, it must be remembered that mol. wt. estimates from gel-filtration studies are rather rough, and valid in principle only to peptides of spherical shape. As the GIA-component may not be a peptide the mol. wt. estimate can only be suggestive.

Former results indicate that the GIA-component may reside deep inside the IF molecule attached by electrostatic bonds (8). If so, the antiviral protein component may protect the GIA-component from outside destruction. Antiviral effect and growth inhibitory activity of leukocyte IF were stable to HCl and to heat. In contrast, the separated GIA-component was destroyed by HCl and activated by heat. On the other hand both activities of IF were destroyed by trypsin and by TCA while separated GIA-component was stable to both. This discrepancy may be explained as follows: when IF is treated with trypsin or TCA, inactive peptides are formed. These peptides are probably still attached to the GIA-component and may block the activity.

It is believed (4, 15) that species specificity of IF depends on specific receptor sites on the cell membrane. Lack of species specificity has been reported (15, 18). In all cases, IF from higher animals was active in cells from lower animals (*C. Chany*, personal communication, june 1976). In agreement with these results a low antiviral titre was found when leukocyte IF was tested in mouse L-F_1 cells. Correlated with this a weak GIA was found. This may suggest that the GIA depends on cell recognition of IF to come into contact with the cells. No species specificity could be detected when the GIA-component was tested in mouse L-F_1 cells. Thus, when the antiviral component is removed from the GIA-component, contact with the cells seems not to depend on specific receptor sites. HeLa cells and L-F_1 cells having a similar growth rate were identically affected. The slower growth rate of HEL cells was less reduced. This confirms the notion that the degree of growth inhibition is somehow correlated with growth rate (9, 17).

The chemical nature of the GIA-component is not known. A negative test for protein (8) by the method of *Lowry et al.* (19) and stability to TCA treatment and to proteolytic digestion may indicate a non protein structure, but do not prove it. Low protein concentration in general and/or few tyrosine

and tryptophane residues will result in a negative Lowry test for protein. Some proteins, especially those containing carbohydrate, are not precipitated by TCA. Proteins lacking arginine and lysine are not digested by trypsin. Like trypsin, pronase hydrolyses peptide bonds, but is not specific for any special amino acid. The concentration of pronase employed in this study (5 μg per ml) was sufficient for the complete destruction of antiviral activity in a purified leukocyte IF preparation (results not shown). Thus, stability to pronase digestion suggests, that the GIA-component is not a protein.

The present study identifies the GIA-component as a distinct molecule different from IF. Further studies of the chemical nature and the mode of action will be required in order to evaluate the significance of the GIA-component in the complex of biological activities exhibited by IF.

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CHARACTERIZATION OF *ENTEROBACTER AGGLOMERANS* (*ERWINIA* SPP.) FROM CLINICAL SPECIMENS

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Ursing, J. Characterization of *Enterobacter agglomerans* (*Erwinia* spp.) from clinical specimens. Acta path. microbiol. scand. Sect. B, 85: 61–66, 1977.

The biochemical reactions of 18 anaerogenic and 45 aerogenic isolates of *Enterobacter agglomerans* are described and used for subdivision in the biogroups suggested by *Ewing & Fife*. The presence of special cultural characteristics (symplasmata and biconvex bodies) was also recorded and was found to be unrelated to formation of gas from glucose. Previous authors have reported such structures in anaerogenic cultures only. The amount of gas produced varied to a great extent between strains and also proved to be dependent on incubation temperature. The results do not support the division of *E. agglomerans* into one anaerogenic and one aerogenic major subgroup. The value of the gelatin liquefaction test for characterization of the species is emphasized.

Key words: *Enterobacter agglomerans* (*Erwinia* spp.); characterization; clinical specimens.

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Received 22.iii.76. Accepted 24.viii.76.

The present study is the result of a re-examination of some strains that were isolated in the laboratory or received for identification over about five years. They were previously considered to be *Erwinia* or atypical members of other genera of *Enterobacteriaceae*. Most of the clinical isolates of *Erwinia* reported by other authors seem to conform to the ubiquitous group of non-pectolytic, usually yellow pigmented bacteria generally known as the herbicola group of genus *Erwinia* (3, 17). *Ewing & Fife* (6) proposed that these bacteria should be included in genus *Enterobacter* as *E. agglomerans*. Different opinions about the taxonomic status of *Erwinia* were extensively reviewed by *Starr & Chatterjee* (20).

In the present paper the biochemical reactions of 63 strains are related to the biochemical pattern of *E. agglomerans* given by *Ewing & Fife* (4, 5). Special attention is paid to the tests that characterize the biogroups suggested by these authors. In addition the occurrence of particular cultural characteristics (i.e. symplasmata and biconvex bodies) has been studied and related to the established biogroups.

MATERIAL AND METHODS

Strains

The investigation was restricted to strains that conformed to the general features of *Enterobacter agglomerans* (4, 5, 6) and that could not be recognized as members of other species within *Enterobacteriaceae*.

Enterobacteriaceae

Of the 63 isolates 27 originated in Malmö General Hospital and 30 in 10 other hospitals in Sweden mainly in the south. The remaining 6 strains were isolated in other countries. The sources of the strains are given in Table 1

TABLE 1 *Sources of Cultures*

Source	No of cultures
Conjunctiva	1
Nose	7
Pharynx	6
Trachea	2
Sputum	14
Wound	20
Urethra	1
Urine	3
Food	3
Water	1
Dust	1
Unknown	4
Total	63

No isolate could be incriminated in connection with severe illness. In two cases a strain was repeatedly isolated in pure culture from a wound From a patient with bronchiectasis a strain was isolated in sputum several times over a year The urinary strains were represented by less than 10 000 bacteria per ml on isolation

Only one strain from each patient was included in the study

Methods

Unless specified below the methods were the same as those described in an earlier communication (21) Most tests were read for 7 days and the incubation temperature was 35 C unless otherwise indicated The tests for motility gas production from glucose and the Voges-Proskauer (VP) test were incubated both at 35 C and at room temperature (RT 22–23 C)

Growth characteristics. The pigmentation of growth was observed for 2 days both on nutrient agar and on the GYCA medium described by *Dye* (2) Incubation temperature 35 C and RT In the nutrient agar cultures kept at RT structures in the centre of colonies were recorded after 2 days with a low power microscope. Symplasmata were studied in hanging drop preparations from nutrient broth cultures after 2 days incubation at RT

Catalase Bacterial growth from nutrient agar was transferred to a glass slide and a few drops of a 3 per cent solution of hydrogen peroxide were added. Negative strains were retested with a 10 per cent solution.

DNase Commercial DNase agar (Oxoid) The test was read after 2 days.

Urease According to *Jessen* (11) with heavy inoculate and reading after 4 hours.

Arginine lysine and ornithine tests according to Møller (18)

Phenylalanine test Method of *Lautrop et al.* (15) The heavy suspensions were incubated at 35 C with and without shaking and reagent was added after 4 hours.

Gelatin liquefaction. Two methods were used 1) Nutrient ferrichloride gelatin according to *Kauffmann* (13) The tubes were incubated at RT and read at intervals up to 60 days. 2) *Kohn's* rapid gelatin test as modified by *Lautrop* (14) with toluene added. The test was read for 7 days.

Pectate liquefaction. Stab cultures in the medium described by *Starr* (19) Sodium polypectate (no 6024 Sunkist Growers Inc. obtained through Otto Vidal & Co, Göteborg, Sweden) was used in 3 per cent (w/v) as originally prescribed, but also in 2 per cent.

RESULTS

General Characteristics

All strains were oxidase negative, catalase positive, gramnegative rods that fermented glucose rapidly with or without formation of gas. They were all negative in the arginine, lysine and ornithine tests. No strain liquefied pectate. Four *Erwinia carotovora* strains were examined as positive controls. They all produced 2–3 mm of liquefaction of the medium and about 10 mm when the pectate content was lowered to 2 per cent.

Growth was good on ordinary media at 35° C and at RT although slightly better at the higher temperature for most of the strains. Motility was generally more pronounced at RT Several strains produced mucoid growth. A more or less intense yellow pigmentation on both media used was recorded for 45 isolates (71 per cent) The colour was generally more pronounced at RT and in a few cultures it was absent at 35 C. Inclusions in colonies in the shape of granular masses and biconvex bodies corresponded very well to *Cruickshank's* description (1) They occurred in the same strains as the

symplasmata. Of the strains exhibiting these features, 36 were yellow pigmented and 4 were considered to be non-pigmented.

TABLE 2. *Numbers of Strains in Different Biogroups* (Ewing & Fife)

Biogroup	No of strains
1	11
2	4
3	1
5	1
6	1
G 1	40
G 2	1
G 3	3
G 4	1
Total	63

Biochemical Reactions

The identification of the isolates was made according to the suggestions of *Ewing & Fife* (4 5) who used the VP indol and nitrate tests for characterizing 7 anaerogenic and 4 aerogenic biogroups. The distributions of strains on biogroups are shown in Table 2. Two anaerogenic biogroups (i.e 4 and 7) were not represented in the material. The biochemical reactions of anaerogenic and aerogenic strains appear in Table 3.

The amount of gas produced from glucose varied to a great extent between strains and was generally more pronounced at 35 C. In 4 strains, however gas was produced rapidly but only at RT one of them showed more abundant growth at the lower temperature. These strains were assigned to aerogenic bio-

TABLE 3. *Behaviour of St ains in Different T sts*

	Anaerogenic 18 strains		Aerogenic 45 strains	
	+	(+)	+	(+)
Glucose gas 35 C	0		41	
Glucose gas RT*	0		40	5
Voges-Proskauer 35 C	9	2	40	1
Voges-Proskauer RT	10	1	40	1
Indole	1		4	
Nitrate reduction	16		44	
Yellow pigment RT	8		37	
Bacross ex bodies RT	7		33	
Symplasmata RT	7		33	
Motility 35 C	15		40	
Motility RT	17		44	
Citrate	16		31	9
Potassium cyanide	9		26	
Malonat	5	1	41	
Beta galactosidase	16		45	
Phenylalanine	0		1	
Gelatin RT	0	14	0	42
Gelatin (Kohn)	9	3	39	3
DNase	2		3	
Arabinose	14	2	44	
Rhamnose	12	3	41	3
Xylose	12	5	43	2

	Anaerogenic 18 strains		Aerogenic 45 strains	
	+	(+)	+	(+)
Fructose	18		45	
Galactose	14	3	45	
Cellobiose	8	1	15	5
Lactose	4	2	11	3
Maltose	8	4	35	10
Melibiose	2		9	
Sucrose	10		44	
Trehalose	15	3	45	
Melezitose	1		0	
Raffinose	1	1	10	
Inulin	0		0	2
Adonitol	1		4	
Dulcitol	0	1	6	
Glycerol	3	10	1	28
Inositol	3	4	0	1
Mannitol	18		45	
Sorbitol	5		6	
Amygdalin	1		0	4
Arbutin	11	4	41	3
Esculin	11	5	40	5
Salicin	12	3	40	4

RT = room temperature.
Positive reaction within 1–2 days incubation +
Positive reaction within 3–7 days' incubation (for gelatin RT up to 8 weeks) (+)
Negative reaction within 7 days' incubation (for gelatin RT within 8 weeks) –
All strains were negative in the following tests Hydrogen sulphide, urease, arginine, lysine, ornithine, sorbose, arabitol and erythritol.

groups although *Ewing & Fife* only considered the behaviour at 35–37° C. One strain, assigned to biogroup 1, was VP positive only at RT and another isolate of the same biogroup had a positive VP reaction only at 35° C.

There was agreement between the results of the two gelatin tests The growth test became positive for 38 cultures within 3–7 days of incubation. Another 5 strains were positive after two weeks and the remaining three strains after 4, 6 and 7 weeks respectively

It appeared that the phenylalanine test became more sensitive after incubation in a shaker For comparison the test was performed on some typical strains of *Escherichia coli* and *Proteus* spp Some of the *E. coli* isolates produced a distinct green colour in 5–10 seconds the *Proteus* suspensions, however, turned very dark green almost immediately after the reagent had been added For the present investigation only a test result comparable to that of *Proteus* was regarded as positive.

All strains fermented trehalose and mannitol most of them also maltose, sucrose and glycerol. Some cultures fermented several carbohydrates in addition. The dulcitol positive strains also attacked cellobiose lactose, melibiose and raffinose Symplasmata or biconvex bodies were not found in cultures *that produced acid from melibiose*

One strain was slightly urease positive at the time of isolation but later negative in repeated tests.

Some strains had a high degree of overall similarity although they differed as to gas formation from glucose

DISCUSSION

As it includes aerogenic strains, the species *E agglomerans* differs from the general concept of the herbicola group of genus *Erwinia* (3 17) However in a study of 54 clinical isolates of *Erwinia von Graevenitz* (8) reported 10 aerogenic strains, and in a similar material of 61 strains, *Gilardi & Bottone* (7) found 6 of them to be gas-forming In the first published material of *Ewing & Fife* (4) the number of aerogenic strains was 69 out of 361 and in a later publication (5) 113 out of 536 The last mentioned authors proposed to divide the species into two major biogroups *on the basis of gas formation and* they remarked that "with a few exceptions, the volumes of gas formed were relatively large (4) This would imply that most of the strains could easily be referred to either of the main biogroups.

In the present collection the difference between anaerogenic and aerogenic strains appeared less clear-cut. The amounts of gas showed a broad variation between strains. Moreover gas production in four isolates proved to be dependent on an incubation temperature lower than 35 C. The proportion of aerogenic strains was high compared with previous reports.

Symplasmata and biconvex bodies are two morphological features originally described in *Bacterium typhi flavum* (1 10 12) later shown by *Graham & Hodgkiss* (9) to be indistinguishable from the herbicola bacteria. *Ewing & Fife* did not report on the presence of these structures in *E agglomerans Von Graevenitz* found symplasmata and biconvex bodies in some of his anaerogenic strains, while they occurred in all the anaerogenic isolates of *Gilardi & Bottone* It appears, however that symplasmata or biconvex bodies have not been described for aerogenic cultures.

During the present investigation symplasmata and biconvex bodies were found in 63 per cent of the total number of strains and in 73 per cent of the gas-forming isolates. Because of the very unusual character of this phenotypical trait, this observation provides further evidence of the similarity of the anaerogenic and the aerogenic strains and tells against the differentiation of the species into *two major subgroups depending on gas forming* ability alone.

The isolates generally exhibited rather less saccharolytic activity than the strains studied by the authors mentioned Other notable dif

ferences concern the phenylalanine reaction and the urease reaction. *Ewing & Fife* found each of these tests positive in 28 per cent of the total number of strains. *Gilardi & Bottone* also had many phenylalanine positive cultures but few urease positive ones. All isolates examined by *von Graevenitz* were on the other hand, negative in both these respects, which is in accordance with the present results. Particularly as regards the phenylalanine test, variation as to technique and interpretation of test results must, however be taken into account.

Apart from the cultural characteristics, the late gelatin liquefaction found in most of the isolates, seem to be of considerable value as a differential test. The less time consuming method of *Kohn* can easily be used as a routine test.

Although inclusion of these bacteria in genus *Enterobacter* seems justified for the time being for reasons given by *Ewing & Fife* (6) the largest differential problem met with during this investigation was *Escherichia coli* strains with negative arginine lysine and ornithine tests. Six such "discarded" isolates lacked the cultural characteristics mentioned above and the ability to liquefy gelatin and they were all cellobiose negative and sorbitol positive. Furthermore *E. coli* antigens could be established in 4 of them. Thus they bore no resemblance to *Leclerc's Escherichia adecarboxylata* (16) which corresponds to *Enterobacter agglomerans* biogroup G 3.

I wish to express my sincere thanks to Dr *H. Lautrop*, Statens Seruminstitut, Copenhagen for his kind interest in this investigation. I am also greatly indebted to Dr *I. Ørskov*, WHO International Escherichia Center, Statens Seruminstitut, Copenhagen for examining some isolates for *E. coli* antigens. My thanks are also due to *Bent Larsen* for excellent technical work.

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groups although *Ewing & Fife* only considered the behaviour at 35–37° C. One strain assigned to biogroup 1 was VP positive only at RT and another isolate of the same biogroup had a positive VP reaction only at 35° C

There was agreement between the results of the two gelatin tests The growth test became positive for 38 cultures within 3–7 days of incubation Another 5 strains were positive after two weeks and the remaining three strains after 4 6 and 7 weeks respectively

It appeared that the phenylalanine test became more sensitive after incubation in a shaker For comparison the test was performed on some typical strains of *Escherichia coli* and *Proteus* spp Some of the *E coli* isolates produced a distinct green colour in 5–10 seconds the *Proteus* suspensions, however turned very dark green almost immediately after the reagent had been added For the present investigation only a test result comparable to that of *Proteus* was regarded as positive.

All strains fermented trehalose and mannitol most of them also maltose, sucrose and glycerol Some cultures fermented several carbohydrates in addition The dulcitol positive strains also attacked cellobiose, lactose melibiose and raffinose Symplasmata or biconvex bodies were not found in cultures that produced acid from melibiose

One strain was slightly urease positive at the time of isolation but later negative in repeated tests.

Some strains had a high degree of overall similarity although they differed as to gas formation from glucose

DISCUSSION

As it includes aerogenic strains, the species *E agglomerans* differs from the general concept of the herbicola group of genus *Erwinia* (3 17) However in a study of 54 clinical isolates of *Erwinia von Graevenitz* (8) reported 10 aerogenic strains, and in a similar material of 61 strains, *Gilardi & Bottone* (7) found 6 of them to be gas-forming In the first published material of *Ewing & Fife* (4) the number of aerogenic strains was 69 out of 361 and in a later publication (5) 113 out of 536 The last mentioned authors proposed to divide the species into two major biogroups on the basis of gas formation and they remarked that "with a few exceptions, the volumes of gas formed were relatively large" (4) This would imply that most of the strains could easily be referred to either of the main biogroups.

In the present collection the difference between anaerogenic and aerogenic strains appeared less clear-cut. The amounts of gas showed a broad variation between strains. Moreover gas production in four isolates proved to be dependent on an incubation temperature lower than 35 C. The proportion of aerogenic strains was high compared with previous reports.

Symplasmata and biconvex bodies are two morphological features originally described in *Bacterium typhi flavum* (1 10 12) later shown by *Graham & Hodgkiss* (9) to be indistinguishable from the herbicola bacteria. *Ewing & Fife* did not report on the presence of these structures in *E agglomerans I on Graevenit* found symplasmata and biconvex bodies in some of his anaerogenic strains, while they occurred in all the anaerogenic isolates of *Gilardi & Bottone* It appears, however that symplasmata or biconvex bodies have not been described for aerogenic cultures.

During the present investigation symplasmata and biconvex bodies were found in 63 per cent of the total number of strains and in 73 per cent of the gas forming isolates. Because of the very unusual character of this phenotypical trait this observation provides further evidence of the similarity of the anaerogenic and the aerogenic strains and tells against the differentiation of the species into two major subgroups depending on gas forming ability alone

The isolates generally exhibited rather less saccharolytic activity than the strains studied by the authors mentioned. Other notable dif

Acta path. microbiol. scand. Sect. B, 85 67–77 1977

ULTRASTRUCTURAL STUDIES ON THE ENDOGENOUS DEVELOPMENT OF *EIMERIA BRUNETTI*

II Microgametogony and the Microgamete

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FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis and Department of Biophysics, Statens Seruminstitut, Copenhagen, Denmark and Department of Biology University of Strathclyde, Glasgow Scotland

Ferguson, D J P., Birch-Andersen, A., Hutchison, W M. & Siim, J Chr Ultrastructural studies on the endogenous development of *Eimeria brunetti*. II Microgametogony and the microgamete. Acta path. microbiol. scand. Sect. B, 85 67–77 1977

The ultrastructural changes of *Eimeria brunetti* which occur during microgametogony in the small intestine of the domestic fowl are described. The development of the trophozoite into the microgametocyte was accompanied by cytoplasmic growth and nuclear division. The developing microgametocytes could be differentiated from developing schizonts because of ultrastructural differences in both cytoplasm and nuclei. The surface area of the microgametocyte was increased by deep invaginations of the limiting membrane. At this developmental stage the nuclei were situated adjacent to this membrane and protrusions which developed, initiated microgamete formation. Two centrioles were positioned between each nucleus and the limiting membrane and were transformed into the basal bodies of the flagella. The flagella grew out from the basal bodies which then entered the protrusions. A mitochondrion and the dense chromatin-containing portion of the nucleus also entered each protrusion The microgametes matured while they were attached to the residual cytoplasm of the microgametocyte they were finally budded off into the parasitophorous vacuole. The microgamete consists of an elongated nucleus which overlaps a mitochondrion towards the anterior of the cell. At this end, a dense plaque (perforatorium) is found together with the 2 basal bodies and their attached flagella 5 microtubules were pparent and run longitudinally from the basal body region I cross section, they were arranged in row of 4 with 1 diagonally opposite only 2 of these microtubules extended to the posterior tip of the microgamete

Key words *Eimeria brunetti* chickens microgametogony microgamete ultrastructure

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Received 16 76 Accepted 13.ix.76

Eimeria brunetti is a pathogenic species of coccidia which infects chickens. Ultrastructural aspects of the endogenous life cycle have not been previously described but recently the asexual development (schizogony) has been investigated (3) This study reports on ultrastructural changes occurring during mi-

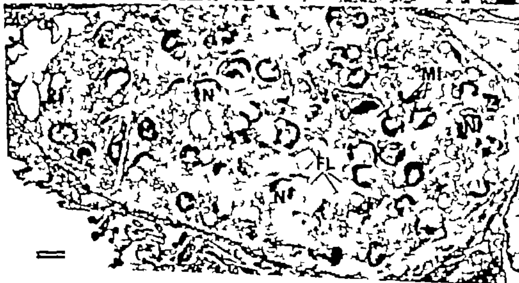
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crogametogony The ultrastructure of the microgamete is also described and the results are compared with the observations reported for other coccidian species.

MATERIALS AND METHODS

The techniques used in the present study have been previously described (3) but can be summarised as follows The chickens examined were killed at 108 1?0 and 144 hours post infection (p.i) Pieces of the small intestine containing parasites were fixed in glutaraldehyde and osmium tetroxide and embedded in Vestopal W Thin sections were examined in the electron microscope after staining with magnesium uranyl acetate and lead citrate The observations were made on material from the same series of infected chickens as used in the previous study (3) and were based on th examination of approx 700 micrographs.

RESULTS

Microgametogony The first evidence of microgametogony was observed at 108 hours p i Numerous microgametocytes at all stages of development were present in the chickens killed at 120 and 144 hours p i.

The host/parasite relationship and the initial development from the merozoite into the trophozoite with accompanying dedifferentiation was similar to that described previously in our report on the development of the schizont (3) As the trophozoite developed into the microgametocyte growth of the cytoplasm was accompanied by nuclear division During these first nuclear divisions it was impossible to distinguish between early schizonts and microgametocytes. In the later stages however it was possible to make a distinction by using ultrastructural differences observed in both cytoplasm and nuclei The cytoplasm of the microgametocyte contained a number of large vacuoles and small dense bodies and further differed from that of the schizont in possessing less rough endoplasmic reticulum but a greater number of free ribosomes In addition the microgametocyte lacked the multi membraneous vacuoles and the dilated Golgi bodies observed in the schizont (cf Fig 1 and Fig 4 of reference 3) The nuclei of the microgametocyte differed from those of the schizont in lacking a distinct nucleolus and showing some of the chromatin aggregated into a number of dense patches at the periphery of the nucleus (cf Figs. 1 & 2 and Fig 4 of reference 3) As illustrated in Text Fig 1 these nuclear changes were accompanied by a reduction in the size of the nuclei in the microgametocyte. Such reduction in size did not occur in the nuclei of the schizont

Nuclear division was similar to that described for the schizont (3) there was an eccentrically located nuclear spindle and the

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Figures 1–16 are all electron micrographs obtained from sections of epithelial cells of the small intestine of chickens infected with *Eimeria brunetti* and illustrate microgametogony and the structure of the microgamete of the parasite

A double bar (=) on a micrograph represents 1 µm and a single bar (—) represents 100 nm.

The following abbreviations are used throughout B Basal Body CE, Centriole CH Chromatin DB Dense Bodies DC Dense Collar FL, Flagella LM Limiting Membrane MI Mitochondrion MP Micropore MT Microtubule N Nucleus NM Nuclear Membrane NU Nucleolus P Plaque (perforatorium) R, Ribosomes RN Residual Nucleus V Vacuoles.

Fig 1 An early microgametocyte showing the large number of nuclei close to the limiting membrane or invaginations of this membrane. The cytoplasm contains large vacuoles, small dense bodies, and mitochondria Nuclear poles are also present (arrows) Generally they are directed towards the centrioles situated between the nucleus and the limiting membrane × 7,500

Fig 2 An enlargement of a nucleus from an organism similar to that shown in Fig 1 The chromatin is distributed in a few dense patches predominately round the periphery of the nucleus. A centriole and many free ribosomes are present in the cytoplasm × 30 000

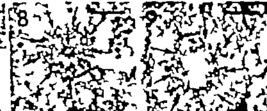
Fig 3 An enlargement of part of an organism similar to that shown in Fig 4 A mitochondrion is seen lying adjacent to a nucleus with condensed chromatin at the periphery A flagellum is seen to protrude into the parasitophorous acuole and to be attached to a basal body × 30 000

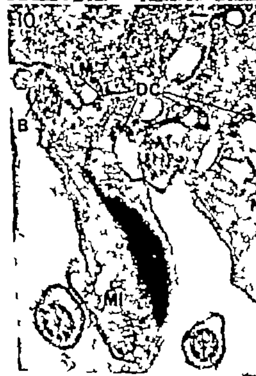
Fig 4 A microgametocyte showing the peripherally condensed chromatin within the nuclei and the presence of flagella. × 7,500


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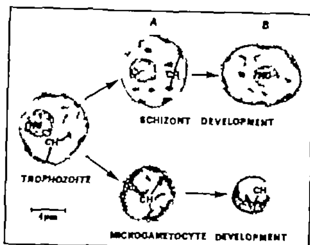

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Text Fig 1 A diagram of the ultrastructural changes observed in the nuclei as the organism develops into either a schizont or microgametocyte. A Chromatin distribution after the initial nuclear divisions B Chromatin distribution after the final *nuclear division.*

nuclear membranes were retained throughout the nuclear division cycle.

The surface area of the microgametocyte was greatly increased by deep invaginations of the limiting membrane which possessed numerous micropores (Fig 7) The micropores were also present at the invaginated regions. As many as three micropores were observed in an area of 0 145 μm^2 The substructure of the micropore (Figs 7 & 11) was *identical to that described for the micropore of the schizont (3) At this stage of development* the nuclei were found lying close to the limiting membrane of the organism, also at the invaginated regions (Fig 1) Normally two centrioles were present between each nucleus and the limiting membrane of the microgametocyte A nuclear pole was often observed directed towards the centrioles.

At this stage, microgamete formation was initiated. Apparently the centrioles developed into the *basal bodies of the flagella this* development appeared to be accompanied by the disappearance of the central microtubule of the centrioles (cf Figs 8 & 9) From each *basal body a flagellum was seen to* protrude into the parasitophorous vacuole and each flagellum was covered by the limiting membrane of the microgametocyte (Figs. 3 & 4)

A *number of microtubules* (usually 4) were observed close to the basal bodies (Fig. 5) On a *number of occasions a dense plaque* was observed between a nucleus and the peripheral membrane of the *microgametocyte* (Fig 6) The plaque may represent the anlage of the perforatorium of the microgamete. A mitochondrion was found to be pre-

Fig 5 Part of a microgametocyte showing two centrioles situated between a nucleus and the limiting membrane. A *row of four* microtubules is present between the centrioles (arrows) × 45 000.

Fig 6 Part of a microgametocyte in which a dense plaque (possibly the perforatorium anlage) can be seen below the limiting membrane. A nuclear pole is directed towards the limiting membrane × 45,000.

Fig 7 A cross section of two micropores *(arrows)* lying adjacent to each other at the surface of a microgametocyte. × 90 000

Fig 8 A cross section of a centriole present in an organism corresponding to that shown in Fig. 1 The nine microtubules (arrows) are arranged in a circle around a single central microtubule × 90,000.

Fig 9 A cross section of a basal body present in a microgametocyte corresponding to that shown in Fig 4 The nine microtubules (arrows) are arranged in a circle but no central microtubule is present. × 90,000

Fig 10 Two longitudinally sectioned developing microgametes. The microgametes are attached to the microgametocyte by their anterior ends. An elongated vacuole (arrow) can be seen in the at*tachment* zone of one of the organisms. Note the collar of dense material close to the limiting membrane in the attachment zone. × 45 000

Fig 11 A cross section of a developing microgamete which shows the mitochondrion and the *dense chromatin part* of the nucleus within a protrusion of the limiting membrane of the microgametocyte. The dense chromatin part of the nucleus *is still attached to the less dense region* which will become the residual nucleus. Note the micropore and the collar of dense material (arrows) at the attachment zone × 45 000

Fig 12 A cross section of a developing microgamete *showing the nucleus, the dense plaque* (perforatorium) the row of four microtubules, and the dense collar at the point of attachment (arrows) × 45 000

ture microgamete budded off into the parasitophorous vacuole and a large residual mass of cytoplasm was left behind. Each microgametocyte was able to produce a large number of microgametes (Fig 13) and evidence for as many as 60 could be seen in a single 50 nm thick section.

The ultrastructure of the microgamete The microgametes were elongated crescent shaped organisms enclosed by a unit membrane The dense nucleus occupied the major part of the microgamete (Fig 15) The nucleus was limited by two closely applied nuclear membranes (Fig 18) Towards the anterior end of the nucleus an elongated mitochondrion was situated adjacent to the nucleus (Fig 14) The mitochondrion possessed several rows of bulbous cristae (Fig 14) At the anterior end of the microgamete the two basal bodies of the flagella were embedded in a dense matrix (Fig 14) There was evidence that this dense material connected the basal bodies (Fig 14) Each flagellum was attached to a basal body and was observed to leave the microgamete close to the anterior end (Fig 14) A dense plaque was found at the anterior tip just below the limiting membrane of the microgamete (Figs. 1? 15 & 16) No definite substructure could be distinguished but a number of short extensions were observed along its length The extensions were located on the side orientated away from the limiting membrane of the microgamete (Fig 15)

A row of four microtubules were present in cross sections. The microtubules were situated between the mitochondrion and the limiting membrane of the microgamete (Fig 17) They were present in sections obtained from the level of the mitochondrial/nuclear overlap and toward the anterior part of the organism (Figs. 16 & 17 In many cases a single microtubule was observed positioned diagonally opposite to this row (Figs. 16 & 17 All these microtubules seem to originate in the basal body region of the microgamete and extend longitudinally through the organism In cross sections taken posteriorly to the mitochondrial nuclear overlap only two microtubules were observed (Fig. 18) and these were found to extend right to the posterior tip of the microgamete Consequently this 4 + 1 microtubular system must consist of three short and two long microtubules. A diagrammatical representation of the ultrastructural organisation of the microgamete is shown in Text Fig 2.

DISCUSSION

The process of microgametogony and the ultrastructure of the microgamete of various coccidian species have been compared to other members of the Sporozoa in reviews by *Scholtyseck et al.* (14) and *Scholtyseck* (13) We will therefore limit our discussion to a comparison of *E. brunetti* with other members of the coccidia.

The ultrastructural characteristics of the cytoplasm and nuclei of the early schizont of *E. brunetti* as described by *Ferguson et al.* (3) differ in a number of ways from those of the cytoplasm and nuclei of the microgametocyte as depicted in the present study This makes it possible to distinguish between the schizont and the microgametocyte prior to the initiation of either merozoite or microgamete formation. The nuclear characteristics described by us were similar to those used by *Ferguson et al* (4) to distinguish between the early schizont and microgametocyte of *Toxoplasma gondii* In addition, the condensation of the chromatin into a single dense mass, as the microgametocyte develops, was similar to that described for the nuclei of the microgametocytes of all other *Eimeria* species which have been investigated (for references see review by *Scholtyseck et al.* 14) The separation of the microgametocyte nuclei into dense chromatin containing microgamete nuclei and less dense residual nuclei would also appear to be characteristic for coccidian microgametogony (14) One exception from this is *E. perforans* described by *Scholtyseck* (12) where it was reported that the entire nucleus entered the developing microgamete.

We have observed that the centrioles in the early microgametocyte appeared to develop

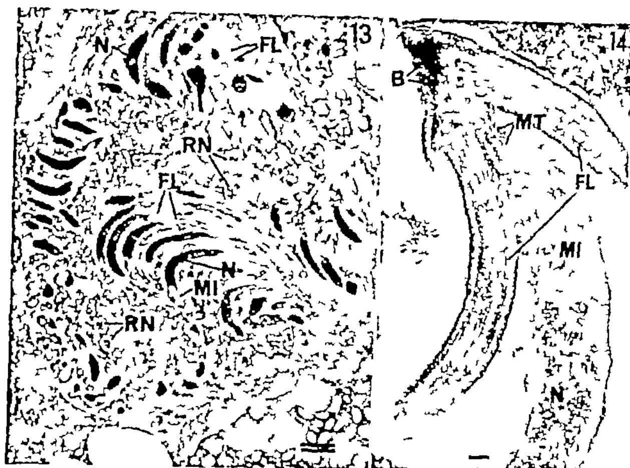


Fig 13 A mature microgametocyte showing the crescent shaped microgametes lying free in the parasitophorous vacuole Note the mass of residual cytoplasm with the residual nuclei × 7,500.

Fig 14 A longitudinal section through the anterior region of a mature microgamete The two flagella are seen to be connected by dense material between their basal bodies. The mitochondrion, the nucleus, and the longitudinally running microtubules are also visible × 45 000

sent adjacent to each nucleus (Fig 3) As *these changes occurred the nuclear chromatin* had condensed into a few large patches (Figs. 3 & 4)

Microgamete formation occurred by a protrusion of the limiting membrane of the microgametocyte in the area directly adjacent to each nucleus (Fig 3) It appeared that the *basal bodies entered the protrusion and were* accompanied by a nucleus and a mitochondrion (Fig 11) At this time the main part of the chromatin had concentrated into a single dense mass at the side of the nucleus closest to the protrusion When the dense area of the nucleus entered the protrusion the less dense region was found to remain attached to it by a narrow channel (Fig 11)

At the base of each protrusion a collar of *dense material was observed below the limit*ing membrane of the microgametocyte (Figs. 10 11 & 12) At maturation of the microgamete the dense part of the nucleus had elongated (Fig 13) This dense region which was now the nucleus of the microgamete separated from the less dense region which re*mained in the cytoplasm of the microgameto*cyte as a residual nucleus (Fig 13) Apparently the microgamete matured while still attached to the microgametocyte The point of attachment was at the anterior end just behind the basal bodies (Fig 10) A membrane bound elongated vacuole was often observed situated close to the point of attachment (Fig 10) At a later stage the fully ma

ture microgamete budded off into the parasitophorous vacuole and a large residual mass of cytoplasm was left behind. Each microgametocyte was able to produce a large number of microgametes (Fig 13) and evidence for as many as 60 could be seen in a single 50 nm thick section

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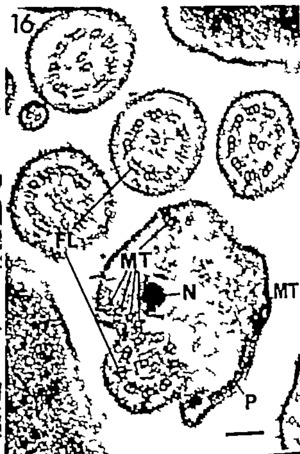
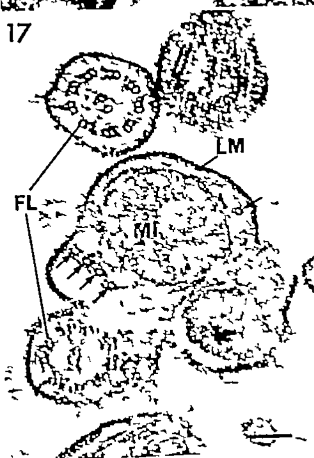
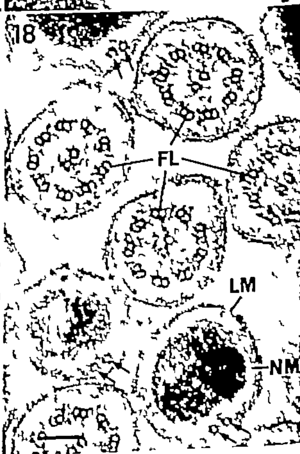
A row of four microtubules were present in cross sections The microtubules were situated between the mitochondrion and the limiting membrane of the microgamete (Fig. 17) They were present in sections obtained from the level of the mitochondrial/nuclear overlap and towards the anterior part of the organism (Figs 16 & 17) In many cases a single microtubule was observed positioned diagonally opposite to this row (Figs. 16 & 17) All these microtubules seem to originate in the basal body region of the microgamete and extend longitudinally through the organism In cross sections taken posteriorly to the mitochondrial nuclear overlap only two microtubules were observed (Fig 18) and these were found to extend right to the posterior tip of the microgamete Consequently this 4 + 1 microtubular system must consist of three short and two long microtubules. A diagrammatical representation of the ultrastructural organisation of the microgamete is shown in Text Fig 2.

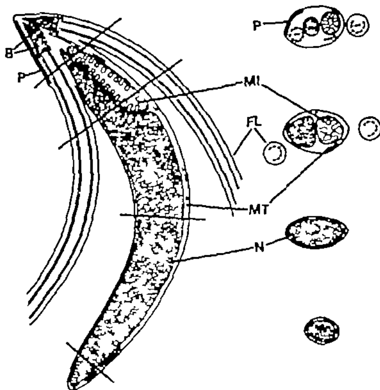
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Text Fig. 2 A diagrammatical representation of the ultrastructural organisation of the microgamete.

Fig. 15 A longitudinal section through the anterior region of a mature microgamete. The dense plaque (perforatorium) with short extensions towards the interior (arrows) is illustrated × 90,000

Fig. 16. A cross section through the anterior region of a microgamete. In this organism the tip of the nucleus, the dense plaque (perforatorium) and the 4+1 arrangement of the microtubules are illustrated. The presence of a microtubule close to the perforatorium is unusual. × 90,000.

Fig. 17 A cross section of a microgamete sectioned slightly posterior to that shown in Fig. 16. At this level the nucleus, the mitochondrion, the flagella, and the 4+1 arrangement of microtubules (arrow) are present × 90,000

Fig. 18 Cross sectioned posterior regions of two microgametes. In each microgamete the nucleus is limited by two closely applied nuclear membranes, and two microtubules of the 4+1 arrangement are present within the limiting unit membrane of the cell (arrow) × 90,000.

into the basal bodies of the flagella. This is in agreement with the observations reported for other *Eimeria spp.* (14) but the apparent change in substructure accompanying this transition has not previously been reported.

The microgametocyte of *E. brunetti* produced numerous microgametes, and as microgamete formation is essentially a surface phenomenon, the surface area was greatly increased by deep invaginations to accomodate the developing organisms. In this respect *E. brunetti* was similar to *E. auburnensis* (6) and *E. maxima* (10) which also produced numerous microgametes. In contrast, *E. perforans* (12), *E. tenella* (8), *E. falciformis* (15) and *Toxoplasma gondii* (4) showed no such increase in surface area but these species produce relatively few microgametes per microgametocyte.

The lack of large polysaccharide granules

in the microgametocyte of *E brunetti* was similar to that reported for *E acervulina* (5) In contrast to this, typical coccidian polysaccharide granules were found to be present in the microgametocytes of the majority of other *Eimeria spp* and of *Toxoplasma gondii* (1 2 4 6 7 8 15 & 16)

The basic ultrastructure of the microgamete of *E brunetti* was similar to that described for the microgametes of other *Eimeria spp* and *Toxoplasma* (see review by *Scholtyseck et al* 14 for references) but a number of differences in details were noted

The basal bodies were embedded in a dense homogeneous matrix, which probably acts as a reinforcement for the perforatorium as well as providing a strong foundation for the attachment of the flagella. Dense connections between the basal bodies of the flagella were observed and it is possible that they have a function in co-ordinating the movement of the flagella

In the microgamete we also observed a dense plaque below the limiting membrane at the anterior end of the organism. This plaque probably represents a reinforcement of the perforatorium and is similar in appearance to the plaques observed at the anterior end of the microgametes of *E auburnensis* (6) *E acervulina* (5) and *Toxoplasma* (4 & 11) We observed no obvious substructure in the plaques but a number of short extensions protruding into the cytoplasm were often resolved At present we are uncertain of what function these extensions may have

The microgametes of *E brunetti* possess two flagella and no evidence of a third flagellum was ever observed. This result is similar to that reported for the microgametes of *E magna E intestinalis E pragensis* and *Toxoplasma* but in most other eimerian species examined so far normally three flagella were observed (see review by *Scholtyseck et al* 14)

We found a row of microtubules running longitudinally through the microgamete Such a row of microtubules has also been reported to be present in certain *Eimeria spp* (14) in *Toxoplasma* (4 & 11) and in *Sarcocystis sp* grown in tissue culture (17) The actual number of microtubules constituting these rows may vary from species to species and may even show variations between individuals of one species (4) The 4 + 1 microtubule arrangement seen in *E brunetti* has not been observed in the other coccidian species investigated Certain of the microtubules, we found could extend to the posterior end of the microgamete. This has also been reported for *E maxima* (9) and *E. falciformis* (15) It has been assumed that the microtubules represent the remains of an additional rudimentary flagellum (14) However such microtubules are also present in *E falciformis* which possesses a third flagellum (15) Therefore in our opinion it is just as likely that the microtubules could have a structural function and for example, provide the microgamete body with the rigidity necessary to withstand the forces created by movement of the flagella.

We are indebted to the *Central Veterinary Laboratory* Ministry of Agriculture, Fisheries and Food, *New Haw Weybridge Surrey* England for supplying a pure sample of oocysts of *E. brunetti* and to *K L. Fennestad* V M.D., Statens Seruminstitut, for provision and maintenance of the chickens. We gratefully acknowledge Mrs. *H Rasn* for technical assistance Miss *A Overgaard* and Mr *F Larsen* for photographic assistance and Dr *R Pontefract* Microbial Research Division, Health & Welfare Ottawa, Canada, for valuable criticism during the preparation of the manuscript.

The work was supported by grants from the *World Health Organisation* Geneva *The Wellcome Trust* and the *British Council*.

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Acta path. microbiol scand. Sect B 85 78-88 1977

ULTRASTRUCTURAL STUDIES ON THE ENDOGENOUS DEVELOPMENT OF *EIMERIA BRUNETTI*

III Macrogametogony and the Macrogamete

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Ferguson D J P., Birch-Andersen A. Hutchison W M & Siim J Chr Ultrastructural studies on the endogenous development of *Eimeria brunetti* III Macrogametogony and the macrogamete. Acta path. microbiol scand. Sect B 85 78-88 1977

The ultrastructural changes occurring during macrogametogony in *Eimeria brunetti* were studied in tissue from the small intestine of infected young domestic fowls. As the macrogametocyte developed, an increase in the volume of the cytoplasm and the nucleus occurred. At this early stage of development the organism was limited by a single unit membrane which possessed a number of micropores. The organism was situated in a parasitophorous vacuole which contained intra vacuolar folds and intra-vacuolar tubules. The wall forming bodies of Type II (WFB II) started to appear before the wall forming bodies of Type I (WFB I) The WFB I developed in the cytoplasmic matrix whereas the WFB II were formed within the cisternae of the rough endoplasmic reticulum apparently in association with Golgi bodies. The mature WFB I were homogeneous, limited by a unit membrane and larger than WFB II which had no limiting membrane but remained surrounded by a membrane of the rough endoplasmic reticulum. Polysaccharide granules were formed in the cytoplasm between strands of the rough endoplasmic reticulum. During maturation the WFB I & II and the polysaccharide granules increased in size and number and as this occurred a number of electron translucent vacuoles were ejected from the surface of the macrogametocyte The mature macrogamete possessed a large nucleus with a nucleolus, a number of multi-membranous vacuoles, mitochondria, and canaliculi. The WFB I & II were located at the cell periphery and the polysaccharide granules further towards the interior of the organism The organism was limited by a unit membrane but during macrogametogony a homogeneous layer had developed which extensively coated the macrogamete surface. Below the limiting membrane two closely applied unit membranes had formed.

Key words *Eimeria brunetti* chickens macrogametogony macrogamete ultrastructure

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Received 27 iii.76 Accepted 15.ix.76

The ultrastructural aspects of the endogenous life cycle of *Eimeria brunetti* have not previously been described. Recently however we have investigated the asexual development (schizogony) and microgametogony of this pathogenic species of coccidia which infects domestic fowls (2 & 3). This study reports on the ultrastructural changes occurring during macrogametogony and the ultrastructure of the macrogamete. The results will be compared with observations reported for other coccidian species.

MATERIALS AND METHODS

The techniques used in the present study have been previously described (2) but can be summarized as follows. The chickens examined were killed at 108, 120 and 144 hours post-infection (p.i.). Pieces of the small intestine containing parasites were fixed in glutaraldehyde and osmium tetroxide and embedded in Vestopal-W. The sections were examined in the electron microscope after staining with magnesium uranyl acetate and lead citrate. The observations on macrogametogony and the macrogamete were made on material from the same series of infected chickens as used in our previous studies and were based on the examination of approx. 500 micrographs.

RESULTS

The first evidence of macrogametogony was observed in chickens killed at 108 hours p.i. and numerous organisms, at all stages of development were observed in the chickens killed at 120 and 144 hours p.i.

The initial development of the infecting merozoite into the trophozoite with accompanying dedifferentiation, was identical to that previously described for the developing schizont (2). However during the formation of the macrogametocyte cytoplasmic growth was not accompanied by nuclear division.

Macrogametocytes were first identified by the presence of large nuclei with prominent nucleoli and amorphous nucleoplasm (Fig. 1). The cytoplasm normally possessed ribosomes, rough endoplasmic reticulum, a number of Golgi bodies, and mitochondria usually located at the cell periphery (Fig. 1). This earliest macrogametocyte was limited by a single unit membrane (Fig. 2) and possessed several micropores. The structure of the micropores (Fig. 6) was similar to that previously described for the developing schizont (2). In the later stages of macrogametogony a large number of micropores could be observed on each macrogametocyte: 4 were observed in a single thin section of one individual.

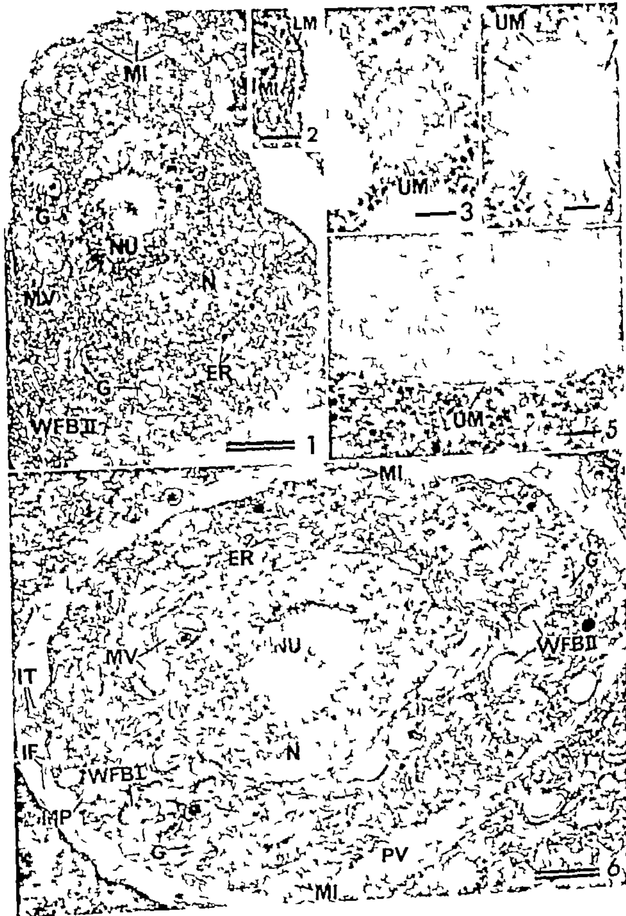
As the macrogametocyte continued to develop, a few wall-forming bodies of Type II* (WFB II) appeared in the cytoplasm (Fig. 1) and at a slightly later stage the wall-forming bodies of Type I (WFB I) were seen (Fig. 6). At this stage a number of multi-membranous vacuoles were also found close to the nucleus (Figs. 1 & 6): these contained some amorphous material. The multi-membranous vacuoles retain their close relationship to the nucleus during macrogamete formation (Fig. 15).

The WFB I were first observed in the cell cytoplasm as spherical structures approx. 244 nm in diameter (D). They had osmophilic contents and were limited by a unit membrane (Fig. 3). Their formation did not appear to be associated with other cytoplasmic organelles. During maturation of the macrogametocyte these bodies retained the enclosing unit membrane (Fig. 5) and could reach a size of approx. 2.05 µm in D (Fig. 15). In the later stages of macrogametogony two forms of the early WFB I were observed: the first was similar to that described earlier (Fig. 3) whereas the other although limited by a unit membrane had contents which consisted of an osmophilic core with a less dense cortex (Fig. 4). It is unknown if a functional difference exists between these two forms or if one is a precursor to the other.

The WFB II first appeared as sponge-like structures (approx. 290 nm) within the cisternae of the rough endoplasmic reticulum (Fig. 8). In close association to this rough endoplasmic reticulum, Golgi body-like struc

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* use the terminology proposed by *Scholtyseck et al.* (14)



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WFBII
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MI

tures were present (Fig 9) As the WFB II developed, their contents became more homogeneous in appearance. No limiting membrane was ever observed, but they remained surrounded by the ribosome covered membranes of the rough endoplasmic reticulum (Fig 10) The mature WFB II could reach a size of approx. 1.24 μm in D

As the macrogametocyte matured, polysaccharide granules became apparent in the cytoplasm. They were normally situated between parallel strands of rough endoplasmic reticulum (Fig 11) In addition, cytoplasm-free canals (canaliculi) delimited by membranes were found. These canaliculi were generally associated in parallel groups and often appeared to radiate from the nucleus into the cytoplasm (Fig. 15) They were retained throughout macrogametogony (Figs. 14 & 15)

As the macrogametocyte developed further an increase in the number and size of the WFB I & II and the polysaccharide granules was noticed. At this time a number of electron translucent vacuoles was seen to be budded off at the periphery of the macrogametocyte (Fig. 14) There was some evidence for these vacuoles being the remains of degenerate mitochondria (Fig. 12) When ejected they were coated by the limiting membrane of the macrogametocyte. At the later stages of macrogametogony pieces of unit membranes had started to form below the limiting membrane of the organism. The structure of the developing macrogametocyte as described in this paragraph is presented in the diagram given in Text Fig. 1

A parasitophorous vacuole which is formed by the host cell contains the developing macrogametocyte it is limited by a unit membrane and possesses a number of intra vacuolar folds. Within the vacuole, a number of intra-vacuolar tubules were found (Figs. 6 & 13) which were approximately 72 nm in D It was impossible to determine the total length of the tubules but portions up to 3.5 μm in length were observed in cross sections their walls appeared to consist of a number of small sub-units (Fig. 13) The tubules were often observed close to the surface of the macrogametocyte but no evidence for fusion with either the limiting membrane of the macrogametocyte or parasitophorous vacuole

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Figures 1 15 are all electron micrographs obtained from sections of epithelial cells of the small intestine of chickens infected with *Eimeria brunetti* and illustrate macrogametogony and the structure of the macrogamete of the parasite.

A double bar (=) on micrograph represents 1 μm and single bar (—) represents 100 nm.

Text Figs. 1 & 2 are diagrammatical representations of the results obtained

The following abbreviations are used throughout AL, Amorphous Layer BM, Basement Membrane C, Canaliculi; ER, Rough Endoplasmic Reticulum EV Electron Translucent Vacuoles G, Golgi Body HMI Host Mitochondria HN Host Nucleus IF Intra-Vacuolar Folds IT Intra-Vacuolar Tubules LM, Limiting Membrane MI Mitochondria MP Micropore MV Multi-Membranous Vacuoles N Nucleus NU Nucleolus PG Polysaccharide Granule PL, Pellicle PV Parasitophorous Vacuole R, Ribosomes UM, Unit Membrane WFB I Wall Forming Bodies of Type I WFB II Wall Forming Bodies of Type II.

Fig 1 An early macrogametocyte showing the nucleus with a large nucleolus, a multi-membranous vacuole, some rough endoplasmic reticulum, and a number of Golgi bodies and mitochondria. Note the WFB II at an early stage of development. × 15,000

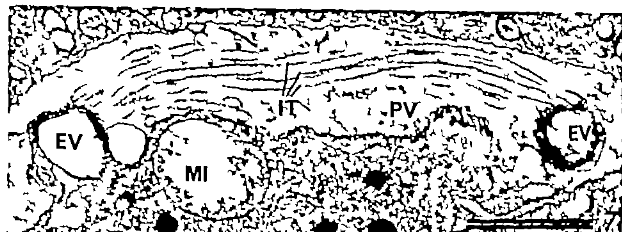
Fig 2 An enlargement of the periphery of the organism show in Fig. 1 The single unit membrane limiting the organism is seen. A mitochondrion is also present. × 90,000

Fig 3 An immature WFB I The amorphous contents of the body are limited by unit membrane. 90,000.

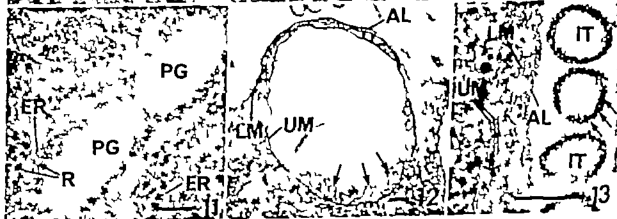
Fig 4 An immature WFB I showing a dense core ith a less dense cortex (arrow) limited by unit membrane × 90,000

Fig 5 Part of a mature WFB I which is 1 7 μm in diameter The body has an amorphous appearance and is limited by unit membrane. × 90,000

Fig 6 An early macrogametocyte is seen in parasitophorous vacuole together with intra-vacuolar folds and intra-vacuolar tubules The organism contains nucleus with prominent nucleolus, few developing WFB I and WFB II number of Golgi bodies, some multi-membranous vacuoles, some peripherally located mitochondria, and considerable amounts of rough endoplasmic reticulum. Note the presence of micropore × 15,000



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was found. Occasionally they appeared to extend between different parts of the macrogametocyte surface, especially in regions where the electron translucent vacuoles were being ejected (Fig 7) The number of intra-vacuolar tubules was found to be greatly reduced in vacuoles with mature macrogametes. The surface of the developing macrogametocytes was extensively coated by an amorphous layer which was approx. 9 nm thick (Fig. 12)

The mature macrogamete, as a rule, had a centrally located nucleus with amorphous nucleoplasm and a conspicuous nucleolus (Fig. 15) In a number of cases a small dense body was also observed in the nucleus (Fig. 15) In the cytoplasm the mature WFB I & II were located at the periphery of the cell and the polysaccharide granules were found interior to them. Multi-membranous vacuoles, canaliculi, rough endoplasmic reticulum and mitochondria were also present in the cell cytoplasm (Fig 15) The surface of the mature macrogamete was smoother than that of the developing macrogametocyte. Although a few of the electron translucent vacuoles were still present, the ejection of these vacuoles which occurred during the earlier stages of macrogametogony did not appear to occur in the mature macrogamete (cf Figs. 14 & 15) The macrogamete was limited by a unit membrane the 9 nm amorphous layer which first appeared on the macrogametocyte was retained on the exterior (Fig 13) Underneath the limiting membrane two closely applied unit membranes were present (Fig 13) The ultrastructure of the mature macrogamete as described in this paragraph is presented in the diagram shown in Text Fig. 2.

Fig. 7 Part of a developing macrogametocyte with intra-vacuolar tubules extending between electron translucent vacuoles which appear to be ejected from the surface of the organism. × 30,000.

Fig. 8 A section through an immature WFB II showing the sponge-like appearance of the body Note the electron translucent space (arrow) between the membrane of the rough endoplasmic reticulum and the body × 90,000

Fig. 9 A developing WFB II The amorphous contents of the body are surrounded by a membrane of the rough endoplasmic reticulum which is closely associated with two Golgi bodies. × 90,000.

Fig. 10 Part of mature WFB II which is 1.2 μm in diameter The amorphous material of the body is separated from the enclosing membrane of the rough endoplasmic reticulum by an electron translucent space (arrow) × 90,000

Fig. 11 Part of an early macrogametocyte showing the polysaccharide granules in the cytoplasm between strands of the rough endoplasmic reticulum 90,000

Fig. 12 Part of a section through the periphery of a developing macrogametocyte A degenerating mitochondrion is seen to protrude from the surface of the macrogametocyte and is thus taking on the appearance of an electron translucent vacuole. Note the remains of few mitochondrial cristae (arrows) and the presence of an amorphous layer on the limiting membrane of the organism × 45,000

Fig. 13 A section through the periphery of mature macrogamete. Two closely applied unit membranes underlying the limiting membrane of the organism are seen. A amorphous layer is present on the exterior of the limiting membrane. Note the banded substructure in the cross sectioned intra-vacuolar tubules × 160,000

DISCUSSION

The host/parasite relationship of the macrogamete of *E. brunetti* is similar to that reported for the schizont and microgametocyte (2 & 3) except for the presence of intra-vacuolar tubules which were absent in the latter two. It has been proposed that these tubules were characteristic for the macrogametes of the genus *Eimeria*, but recently it has been reported that only a small percentage of macrogametes of *E. labbeana* contains them (17) The tubules are absent in *E. ferrisi* (1) and they are also absent in *Isospora felis* and *I. rivolta* (9) and *Toxoplasma gondii* (4 & 10) The diameter of the tubules in *E. brunetti* is within the range reported for other *Eimeria spp* (13) The wall is composed of a number of sub-units and this is similar to what has been reported for *E. maxima* *E. perforans* and *E. labbeana* (6 13 & 17) It has been proposed that these tubules function in the transport of material between

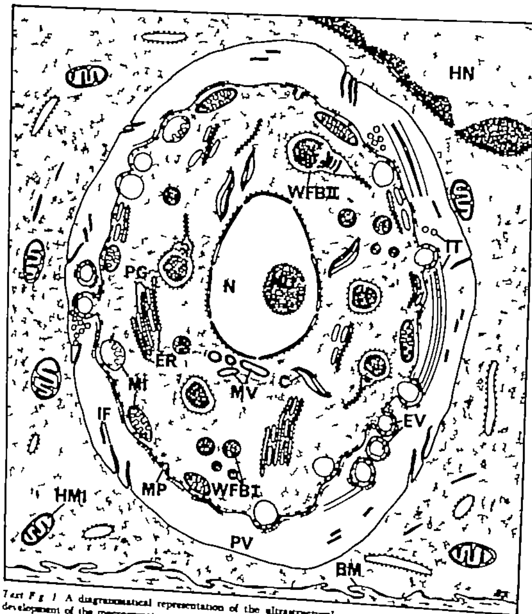


Fig 14 Part of a developing macrogametocyte in which the nucleus with its nucleolus, a number of WFB I and WFB II some polysaccharide granules, canaliculi, and mitochondria can be seen. Note the electron translucent vacuoles at the periphery of the macrogametocyte. × 7,500

Fig 15 Part of a mature macrogamete showing the centrally located nucleus with its nucleolus and a small dense body (arrow) The WFB I and WFB II are present in the peripheral cytoplasm with the polysaccharide granules more centrally located. Canaliculi, multi-membranous vacuoles and mitochondria are also present Note the smooth appearance of the surface of the macrogamete. × 7,500

the host cell and the macrogamete and direct connections between host cell tubules and macrogametocyte have been reported by *Scholtyseck et al* (13) *Michael* (8) and *Varghese* (17) Our observations that the number of intra vacuolar tubules is larger in the developing macrogametocyte could support the nutritional hypothesis. It is reasonable to expect that the nutritional needs of the developing macrogametocyte would be greater than those of the mature macrogametes.

During macrogametogony in *E brunetti* an initial degeneration of the inner layer of the merozoite was observed Thus the macrogametocyte was at this stage limited by a single unit membrane, but eventually a regeneration of two unit membranes in the later stages of macrogametogony occurred. The macrogamete was consequently limited by three unit membranes. This degeneration and regeneration of membranes is similar to that reported for *E intestinalis* (15) *E acervulina* (5) and *E ferrisi* (1) The structure of the limiting membranes of the macrogametes of other *Eimeria spp* have been reviewed by *Chobotar et al* (1) and *Varghese* (17) The finding of an amorphous layer coating the macrogamete has not previously been observed in any *Eimeria spp.*, but it bears some resemblance to the first layer of the oocyst wall of *Toxoplasma* (4) The origin

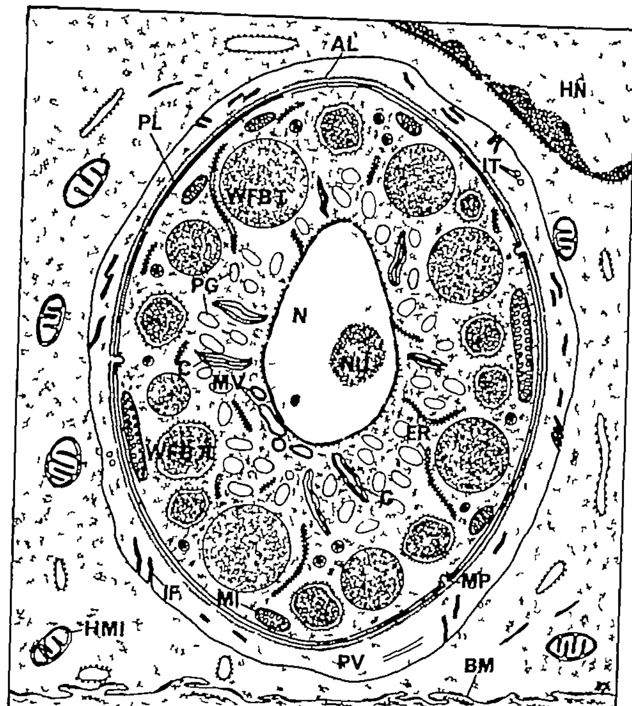


Text Fig. 1. A diagrammatical representation of the ultrastructural organisation at late stage in the development of the macrogametocyte

and function of this coating layer could not be determined.

The apparent formation of the WFB I in the cytoplasmic matrix of the cell has also been reported for *E. ferris* (1). The fact that the bodies are limited by a unit membrane and have homogeneous contents is typical for *Eimeria spp.* and *Toxoplasma* (see *Scholtyseck et al.* (13) for references). In *E. brunetti* the WFB I were larger than the WFB II which is also the case in *E. labbeana* (17) *E. tenella*, *E. stiedae*, *E. perforans* and *E. maxima* (13).

The formation of the WFB II seems to be associated with the rough endoplasmic reticulum and Golgi bodies of the organism. This is



Text Fig 2 A diagrammatical representation of the ultrastructural organisation of the mature macrogamete

similar to that reported for other *Eimeria spp* (11 & 13) In *E brunetti* the WFB II have a homogeneous appearance and are surrounded by a membrane of rough endoplasmic reticulum as evidenced by the presence of ribosomes on the cytoplasmic side of the membrane. This is also the case in *E falciformis* (12) *E magna* (16) *Isospora spp* (9) and *Toxoplasma gondii* (4 & 10) but it is different from the structure of the WFB II of other *Eimeria spp* which present a loose or mesh like appearance (13)

The multi membranous vacuoles in the macrogamete differ from those present in the schizont (2) because of their close relationship with the nucleus. Similar vacuoles have

been observed close to the nucleus in the macrogametes of *E. magna* (16) *E. acervulina* (7) *E. labbeana* (17) and *Toxoplasma* (4). It has been proposed by *Michael* (7) and *Ferguson et al.* (4) that they are formed from the nuclear membranes. If so, it is possible that they contain nuclear material which is unnecessary for the later development of the oocyst.

The structure of the polysaccharide granules and their formation during macrogametogony is similar to that reported for other *Eimeria spp.* (13). Lipid inclusions were absent in the macrogametes of *E. brunetti*.

Electron translucent vacuoles appeared to be ejected from the surface of the macrogametocyte. Such vacuoles have not previously been observed in members of the coccidia. They appear to be derived from degenerating mitochondria and may thus represent a means of waste disposal. In schizogony and microgametogony residual masses of cytoplasm are left behind when the mature organisms are budded off; these residual masses could contain the waste material present. In macrogametogony, however, the complete macrogametocyte develops into the macrogamete; therefore, any toxic material or other waste products have to be ejected and a possible function of the vacuoles could be to provide a means for this ejection.

We are indebted to the *Central Veterinary Laboratory*, Ministry of Agriculture, Fisheries and Food, New Haw, Weybridge, Surrey, England, for supplying a pure sample of oocysts of *E. brunetti*, and to *K. L. Fennestad*, V.M.D., Statens Seruminstitut, for provision and maintenance of the chickens. We gratefully acknowledge Mrs. *H. Rat* for technical assistance, Miss *A. Osterguard* and Mr. *F. Lauritsen* for photographic assistance.

The work was supported by grants from the *World Health Organization*, Geneva, *The Wellcome Trust* and the *British Council*.

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CHEMICAL MODIFICATION AND CHARACTERIZATION OF ENTEROTOXIN FROM *CLOSTRIDIUM PERFRINGENS* TYPE A

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Granum, P. E. & Skjelkvåle, R. Chemical modification and characterization of enterotoxin from *Clostridium perfringens* type A. Acta path. microbiol. scand. Sect. B, 85: 89–94, 1977.

Enterotoxin from *Clostridium perfringens* type A has been purified. The enterotoxin was shown to be heat-labile, but re-activation of heat treated enterotoxin did occur down to an activity of 15 per cent of the native enterotoxin. The molecular weight was shown to be 34,000 by ultracentrifugation, and the molecular weight did not change significantly after treatment with 0.1 M β-mercaptoethanol and 6 M guanidine hydrochloride. It was concluded that the enterotoxin consists of one single polypeptide chain. The enterotoxin did not lose any activity after treatment with iodoacetic acid and iodoacetamide, but a complete loss of activity was observed after succinylation.

Key words: *Clostridium perfringens*; enterotoxin; chemical modification.

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Received 4.ix.76. Accepted 13.ix.76

A heat labile enterotoxin has been purified from certain strains of *Clostridium perfringens* during sporulation (7, 21). The toxin induces fluid accumulation in ileal loop tests in animals (9), and diarrhea in humans involved in *C. perfringens* food poisoning (10).

Despite the relatively mild symptoms and rapid recovery of humans after this type of food poisoning, it has been shown that the toxin may cause denuding of rat and rabbit ileal villi (14, 16). A disturbance in the intestinal electrolyte and water balance has also been reported (15).

The enterotoxin is a pure protein with a molecular weight of approximately 34,000 and a pI 4.3. Further details about the molecular structure and mode of action of the toxin are still lacking. In this report an attempt will be made to elucidate the effects of chemical modification on the biological and serological activity and molecular structure of the toxin.

MATERIALS AND METHODS

Production and Purification of Enterotoxin

C. perfringens type A strain NCTC 8239 was obtained from C. L. Duncan, Food Research Institute, University of Wisconsin, Madison, U.S.A. Stock cultures of this strain were maintained in cooked meat medium, and used for enterotoxin production in Duncan and Strong (DS) sporulation medium (4) throughout the study.

Cell extracts from cultures grown in DS medium for 8 h at 37° C were prepared according to *Stark & Duncan* (21). The enterotoxin was purified by a modification of the method described by *Sakaguchi et al.* (18). After ammonium sulfate precipitation and differential solubilization, the dissolved proteins were dialyzed against 0.003 M Na-

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Calculations were made according to *Yphantis* (22). A partial specific volume of 0.73 ml/g was used for the enterotoxin (8).

Amino Acid Analysis

The amino acid composition of the enterotoxin was determined with a Biocal BC 200 automatic amino acid analyzer. Hydrolysis under vacuum was performed in 6 N HCl at 108 C for 24 h. Tryptophan was determined by the method of *Matsubara & Sasaki* (15) and cysteic acid after *Hirs* (11).

RESULTS

Heat Treatment and Reactivation

The ion-exchange chromatography on Cellex T gave enterotoxin of 98–99 per cent purity in polyacrylamide disc gel electrophoresis as determined by densitometric tracing (Gelman DCD-16) (Fig. 1).

The enterotoxin was found to be heat labile, losing 90 per cent of its activity after 1, 5 and 15 min at 60 C, 57 C, and 55 C, respectively. At 50 C and 53 C no significant loss of activity was observed within a period of 30 min. After 120 min, 25 per cent activity was lost at 50 C and 35 per cent at 53 C (data not shown).

Reactivation of the enterotoxin which was heat-treated at 55 C for 1, 5 and 15 min is shown in Fig. 2. The toxicity of the enterotoxin, heat treated for 1 min, increased relatively rapidly from 30 per cent to an apparently stable level of about 50 per cent after

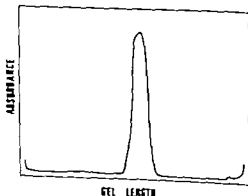


Fig. 1. Densitometric tracing of stained acrylamide gel after electrophoresis of purified enterotoxin (100 µg).

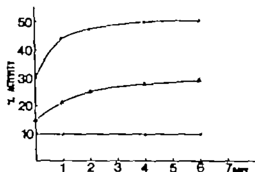


Fig. 2. Reactivation curves of purified enterotoxin heat treated at 55 C for 1 min, ●; 5 min, △ and 15 min, ○. The activity is given in per cent of enterotoxin activity before heat treatment.

4 days. Correspondingly the enterotoxin heat treated for 5 min increased its biological activity more slowly from 15 per cent passing 28 per cent after 6 days without having reached a stable level, and enterotoxin heat treated for 15 min showed no reactivation within the period of measurement.

Effects of Chemical Modifications

The treatment of the enterotoxin with iodoacetamide and iodoacetic acid did not show any significant loss of biological activity. The serological activity of enterotoxin, treated with iodoacetamide and iodoacetic acid, was also unaltered (Fig. 3).

Complete loss of biological activity in the guinea pig skin test was observed after succinylation of the enterotoxin. Immunodiffusion showed no change in immunological identity after succinylation, and the immunological reactivity in immunoelectrophoresis was not affected (Fig. 3).

Analytical Ultracentrifugation

The molecular weight was determined from sedimentation equilibrium experiments with purified enterotoxin, enterotoxin denatured with 6 M guanidine hydrochloride, and enterotoxin denatured with 6 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol. All three plots of fringe displacement against radial distance were

phosphate buffer pH 8.0 and subjected to ion-exchange chromatography on Cellex T (Biorad Laboratories) as previously described (21).

Protein Determination

Protein determination was done by the Lowry method with bovine serum albumin (BSA) as standard (12).

Production of Antisera

Antiserum against purified enterotoxin was produced by immunization of rabbits as described previously (21).

Guinea Pig Skin Test

Erythemal activity in terms of erythemal units was assayed by the guinea pig skin test as described by *Stark & Duncan* (20). In control samples the toxin was neutralized with specific antienterotoxin serum from rabbit.

Disc Gel Electrophoresis

Disc gel electrophoresis was performed on 7.0 per cent acrylamide gels with pH 8.5 tris-(hydroxymethyl) aminomethane-glycine as the running buffer (2). The gels were loaded with 50-250 μg purified or partially purified enterotoxin protein. The gels were stained with Coomassie brilliant blue R 250.

Immunodiffusion and Electroimmunodiffusion

Immunodiffusion studies were performed on Petri dishes with an agar layer consisting of 1 per cent Nobel agar (Difco), 1 per cent NaCl and 1:10 000 merthiolate.

Serological quantitation of enterotoxin was done by electroimmunodiffusion (3). Sample volumes were 8 μl and electrophoresis were performed in 0.5 per cent Litex agarose gel (Litex, Copenhagen) containing antibodies diluted 1:800. Field strength in the gel during electrophoresis was 4 volt/cm.

Heat Stability and Reactivation of Heat-treated Enterotoxin

Purified enterotoxin (1 mg/ml) in 0.02 M phosphate buffer pH 6.8 was added to small test tubes (10 × 90 mm) which were heated in water baths at 50, 53, 55, 57 and 60 C from 1 to 120 min. To stop the reactions, the test tubes were placed in an ice bath. The enterotoxin activity was tested by the guinea pig skin test.

Enterotoxin which was heat treated at 55 C for 1, 5 and 15 min was stored at 20 C for 1 to 7 days. Reactivation was assayed by the guinea pig skin test.

Treatment with Iodoacetic Acid and Iodoacetamide

Studies were made with iodoacetic acid and iodoacetamide after the method of *Bern & Rack* (1). The reaction mixtures contained 0.25 mg/ml of enterotoxin, 2 μmoles/ml of iodoacetic acid or iodoacetamide, dissolved in 0.02 M phosphate buffer pH 8.0. The reactions were conducted at 20 C for 16 h. After being dialyzed against 0.02 M phosphate buffer pH 6.8 overnight, the enterotoxin activity was tested by immunodiffusion, electroimmunodiffusion, and the guinea pig skin test.

Succinylation of the Enterotoxin

To 5 ml enterotoxin (2 mg/ml) 10 mg solid succinic anhydride was added. The reaction was performed in 0.02 M Na-phosphate buffer pH 7.6, and the pH was maintained at 7.6 by the addition of 0.5 N NaOH (17). After 30 minutes another 10 mg of solid succinic anhydride was added. The reaction mixture was then allowed to stand for two hours at room temperature. After being dialyzed against 0.02 M phosphate buffer pH 6.8 overnight, the enterotoxin activity was tested by immunodiffusion, electroimmunodiffusion, and the guinea pig skin test.

Denaturation Studies with Guanidine Hydrochloride

Purified enterotoxin was added to an 8 M solution of guanidine hydrochloride, making the final concentration of guanidine hydrochloride 6 M and the final concentration of enterotoxin 0.45 mg/ml. The final concentration of the phosphate buffer was 0.1 M (pH 6.8).

An experiment was also performed with purified enterotoxin and 6 M guanidine hydrochloride with 0.1 M β-mercaptoethanol. In this experiment 0.1 M phosphate buffer pH 6.0 was used. The solutions were dialyzed for 24 h against buffers of the same composition, except for enterotoxin.

Analytical Ultracentrifugation Experiments

Sedimentation equilibrium experiments were performed at 22 C in a Beckman Analytical Ultracentrifuge Model E using a double beam photoelectric scanner at 280 nm. The final enterotoxin concentration was 0.45 mg/ml.

The molecular weights were determined at a rotor speed of 20 000 rev/min.

Three samples were analyzed:

1. Enterotoxin in 0.1 M phosphate buffer pH 6.8.
2. Enterotoxin and guanidine hydrochloride in 0.1 M phosphate buffer pH 6.8.
3. Enterotoxin, β-mercaptoethanol and guanidine hydrochloride in 0.1 M phosphate buffer pH 6.0.

Calculations were made according to *Yphantis* (22). A partial specific volume of 0.73 ml/g was used for the enterotoxin (8).

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The amino acid composition of the enterotoxin was determined with Bocal BC 200 automatic amino acid analyser. Hydrolysis under vacuum was performed in 6 N HCl at 108° C for 24 h. Tryptophan was determined by the method of *Matsubar & Sasaki* (15) and cysteic acid after *Hirs* (11).

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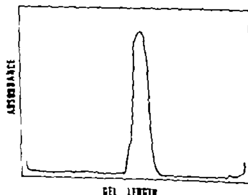


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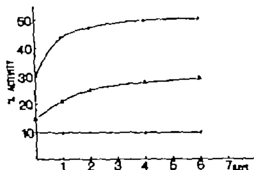


Fig. 2. Reactivation curves of purified enterotoxin heat treated at 55 C for 1 min, ●, 5 min, △ and 15 min, ○. The activity is given in per cent of enterotoxin activity before heat treatment.

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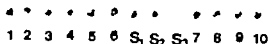


Fig 3 Immunoelectrophoresis of purified and chemically modified enterotoxin. Sample volumes were 8 μl Protein concentration for the standard purified enterotoxin was 5 10 and 20 μg/ml protein for S_1 S_2 and S_3 respectively Concentration for all other samples were 16 μg/ml

1 & 2 enterotoxin treated with iodoacetic acid.
3 & 4 enterotoxin treated with iodoacetamide.
5 & 6 purified enterotoxin.
S_1–S_3 standard purified enterotoxin
7 & 8 enterotoxin treated with succinyl anhydride
9 & 10 purified enterotoxin.

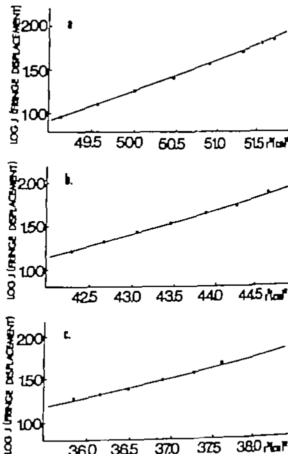


Fig 4 Sedimentation equilibrium experiments in a Beckman Analytical Ultracentrifuge, Model E, with the use of double beam photoelectric scanner at 280 nm.

a. Purified enterotoxin. Protein concentration 0 45 mg/ml.
b Purified enterotoxin with 6 M guanidine hydrochloride Protein concentration 0 45 mg/ml.
c. Purified enterotoxin with 6 M guanidine hydrochloride containing 0 1 M β-mercaptoethanol. Protein concentration 0 45 mg/ml

linear indicating that the preparations were homogenous (Fig 4)
The molecular weights were calculated to be 34 000 for the purified enterotoxin 40 000 for enterotoxin with 6 M guanidine hydrochloride and 35 000 for enterotoxin with 6 M guanidine hydrochloride containing 0 1 M β mercaptoethanol

Amino Acid Analysis

The amino acid composition of the enterotoxin is given in Table 1 The results are in good agreement with those of *Hauschild et al* (8) The only important significant difference is the result of cysteic acid. In this investigation it was found that the enterotoxin contained only one cysteic acid residue per molecule, compared to two cysteic acids found in the work of *Hauschild et al* (8)

DISCUSSION

The enterotoxin (1 mg/ml) was relatively stable up to 120 min at 50 C and 53 C, but had a rapid loss of biological activity at higher temperatures. Reactivation experiments with enterotoxin showed that the protein structure is not irreversibly destroyed before the biological activity of the enterotoxin is less than 15 per cent of the activity before heat treatment At 10 per cent biological activity the ability for reactivation is lost. This may indicate that the enterotoxin poss-

TABLE 1 *Amino Acid Composition of Clostridium perfringens Enterotoxin*

Amino acid	Per cent amino acid residues	No. of residues based on molecular weight 34000
Lysine	5.45 ± 0.08	16.8
Histidine	1.08 ± 0.03	3.5
Arginine	2.14 ± 0.02	6.6
Aspartic acid	14.51 ± 0.12	44.6
Threonine	6.81 ± 0.06	21.0
Serine	11.01 ± 0.12	33.9
Glutamic acid	9.46 ± 0.04	29.1
Proline	2.96 ± 0.17	9.1
Glycine	7.07 ± 0.10	21.8
Alanine	5.27 ± 0.13	16.2
Cysteic acid	0.36 ± 0.05	1.1
Valine	5.68 ± 0.35	17.5
Methionine	0.77 ± 0.00	2.4
Isoleucine	7.40 ± 0.22	22.8
Leucine	9.36 ± 0.18	28.8
Tyrosine	5.78 ± 0.06	17.8
Phenylalanine	3.76 ± 0.08	11.6
Tryptophan	1.12 ± 0.08	3.4

esses some biological activity after having lost its original structure.

Blocking of SH-groups by sulfhydryl reagents had no effect on the biological or immunological activity of the enterotoxin. This is not surprising as the amino acid analysis shows that the enterotoxin only contains one cysteic acid residue per molecule.

Succinylation of the enterotoxin at pH 7.6 resulted in complete loss of biological activity. Succinic anhydride shows a strong preference for amino groups but may also react with hydroxyl and sulfhydryl groups (6). Succinylation of tyrosine can be followed by measuring the decrease in absorbance at 278 nm, and the formed succinyl tyrosin has been reported to spontaneously decompose after 3 to 4 h. No change in absorbance at 278 nm was observed, so this possibility can be excluded. Succinylation of the hydroxyl groups on serine and threonine is reported for the free amino acids under suitable conditions (26–29 per cent for serine and 3–6 per cent for threonine) and cannot be totally excluded (6).

Since succinylation inactivates the enterotoxin without changing the immunological activity, it may be suggested that the shape of the protein is essentially unaltered after succinylation, and that free amino groups are most probably essential for the proteins toxicity.

The molecular weight of the enterotoxin was calculated to be 34,000 from the analytical ultracentrifugation, which is in agreement with earlier studies of the enterotoxin (7, 8, 21). In previous studies of the enterotoxin in sodium dodecyl sulphate (SDS) gel electrophoresis it was found that the enterotoxin showed anomalous behaviour. Upon SDS gel electrophoresis under reducing and dissociating conditions, molecular weights ranging from 34,000 to 175,000 with increasing increments in multiples of 17,000 were noted (5, 19), and it was indicated that the enterotoxin may have two basic subunits with a molecular weight of 17,000.

Denaturation of the enterotoxin with guanidine hydrochloride and treatment with β-mercaptoethanol did not result in significant changes of the molecular weight.

It is therefore concluded that the enterotoxin monomer has a molecular weight of 34,000 and that the enterotoxin consists of one single polypeptide chain.

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Acta path. microbiol. scand. Sect. B, 85: 95–102, 1977

EXPERIMENTAL TOXOPLASMOSIS IN MICE AND RABBITS

Virulence and Cyst Formation of Toxoplasma gondii

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Pettersen, E. K. Experimental toxoplasmosis in mice and rabbits. Virulence and cyst formation of *Toxoplasma gondii*. Acta path. microbiol. scand. Sect. B, 85: 95–102, 1977.

Avirulent *Toxoplasma gondii* were made virulent by passages in mice, and then attenuated by storage. In contrast to the avirulent parasites, attenuated organisms did not appear to form cysts in mice and rabbits, although parasites could be isolated from such animals as long as three months after their inoculation. Rabbits infected with parasites of attenuated virulence had high antibody levels, and survived challenge with virulent *Toxoplasma gondii*. Cysts were not detected in the brains of such rabbits following this second infection. By following the generation of *Toxoplasma gondii* in mouse peritoneal cavities, it was found that attenuated parasites resembled avirulent more than virulent organisms. Although the effect of attenuation was mainly on the parental generation of parasites, some genetic effect seemed to be involved as well.

Key words: Experimental toxoplasmosis; *Toxoplasma gondii*; virulence; cyst formation.

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Received 22 [illegible] 76. Accepted 29 ix 76.

Since the discovery of *Toxoplasma gondii* this protozoan parasite has been isolated from a vast number of hosts. The different strains thus obtained have been found to vary only with respect to their virulence for test animals such as mice (*Kaufman et al.* 1958). If the mice are still alive three weeks after inoculation with *Toxoplasma gondii* they have most likely survived the infection. The inoculated strain is then considered to be avirulent and, if the surviving mice have positive dye test titres, they are considered to be chronically infected with encysted parasites ([illegible] 1959). The presence of cysts can be demonstrated in mouse brains by microscopy or by subinoculation into normal mice with resultant toxoplasmosis.

In order to survive an infection with virulent *Toxoplasma gondii* the mice have to be protected. Following the use of chemotherapy *Nakayama & Matsubayashi* (1961), *Frenkel* (1954), *Beverly & Fry* (1957), *Medlvedt* (1964) and *Lai et al.* (1974) reported cysts, carriers or chronicity among mice surviving infections with the virulent RH strain. It appeared that there was a delicate balance between too much and too little treatment which allowed both mice and parasites to survive.

In order to find a more reproduceable way of forming cysts in mice with virulent *Toxoplasma gondii* it was decided to attempt attenuation of virulence before inoculation. In order to avoid possible strain differences it was decided to compare the cyst forming abil-

ity of an avirulent strain with that of the same strain made virulent.

In this paper "virulent parasite" refers to one with virulence equal to that of the RH strain killing all inoculated mice. An "avirulent parasite" is one that does not kill all, if any inoculated mice. An "attenuated parasite" is one which was virulent and became avirulent following some kind of treatment.

MATERIALS AND METHODS

The virulent RH strain of *Toxoplasma gondii* is maintained in this laboratory by weekly passage in mice of peritoneal exudate. Each mouse is inoculated with approximately 20 000 parasites. The exudate is collected four or five days after the inoculation.

Strain 119 had been isolated from a pig by *Work* (1968) and Strain 178 by *Sim* in 1971 from a human patient. These two avirulent strains were maintained in mice by peritoneal inoculation two or three times a year of brain tissue containing cystozoites (*Hoare* 1972).

Four week old white mice bred at Statens Seruminstitut weighing 18–20 g were used in all experiments. Each mouse received a 0.2 ml inoculum intraperitoneally. The rabbits also were bred at the institute. They were four months old and weighed approximately 2 kg at the time of inoculation. With the exception of the subcutaneous inoculation of lysed parasites, rabbits were infected by coating the eye with a cotton swab saturated with a suspension of viable parasites (*Kåss* 1954, *Sim* unpublished work). The volume applied in this way was 20–40 µl.

The effect of inoculation of cystozoites and endozoites (*Hoare* 1972) into mice was determined by counting the number of parasites present in peritoneal fluids. For this purpose the mice were killed by gaseous carbon dioxide. The peritoneal cavity was rinsed with approximately 0.5 ml of isotonic saline if an exudate was present in adequate volume, it was aspirated undiluted. The effects of inoculating mice and rabbits with *Toxoplasma gondii* were further determined by the Sabin Feldman dye test (*Aagaard* 1960, *Sabin & Feldman* 1948). For this purpose bloods were drawn from the tails of mice and the ears of rabbits approximately three weeks after inoculation.

Examination for cyst formation was usually done three months after inoculation. It required the removal of brains from mice and rabbits. Unstained smears of the brain were examined microscopically, the remainder of the brain was homogenized in a Potter Elvehjem tissue grinder. Homogenized mouse brain was inoculated into normal mice after removal of the coarser particles. Homogenized rabbit brain was treated with a pepsin solution in dilute hydrochloric acid for 20 minutes at 37 C (*Jacobs & Melton* 1957). The pepsinized suspension was centrifuged at 4 C and the sediment was resuspended in isotonic phosphate buffer (*Work* 1967) and inoculated into normal mice after the removal of coarser particles.

The virulence of Strain 119 was increased by rapid passages in mice. Liver and spleen (*Jacobs* 1956) were removed five days after the inoculation of cystozoites and suspended in phosphate buffer with a Potter Elvehjem tissue grinder. Coarse particles were allowed to settle for a few minutes and the supernate was inoculated intraperitoneally into normal mice without further delay. This procedure was repeated every fourth or fifth day until peritoneal exudate was found in the mice. The exudate was then used for subsequent passages rather than the liver and spleen homogenate. The virulent Strain 119 was maintained as described for the RH strain.

In order to attenuate the virulence of parasites, they were left for different time intervals at 37 C. The medium was either undiluted exudate or exudate diluted with an equal volume of phosphate buffer containing 10 000 I.U. of penicillin per ml. Another attenuation of virulence was attempted by suspending the parasites in phosphate buffer containing a high molecular weight fraction of an extract of *Toxoplasma gondii*. This fraction was obtained by gel filtration of the *Toxoplasma* extract on a column packed with Sepharose CL-6B (Pharmacia Fine Chemicals, Sweden). Such a high molecular weight fraction was reported to be an inhibitor of a penetration enhancing factor (*Lycke et al.* 1975).

The phosphate buffer used in these experiments was 0.05 M with respect to sodium phosphate, 0.1 M with respect to sodium chloride, and 0.003 M with respect to sodium azide. The pH was adjusted to 7.4.

RESULTS

The increase in virulence of Strain 119 is illustrated in Fig. 1 where the points symbolize death of single mice in each passage. From the ninth passage onwards peritoneal exudate was formed and all of the mice died rapidly. After 13 passages the virulence of Strain 119 was comparable to that of the RH strain (Table 2).

The appearance of virulent Strain 119 *Toxoplasma* was investigated in relation to the number of endozoites inoculated. The

TABLE 1 *Inoculation into Normal Mic of Brain Tissue from Mice and Rabbits Surviving Infection with Virulent* Toxoplasma gondii

Number animals	Inoculum	Weeks after inoculation	Reciprocal dye test titre	Number of mice died/inoculated
5 mice	a)	7	50	15/15
5	a)	11	50	0/25
10	a)	12	10	0/50
5	)	15	10	0/15
5	b)	8	50	15/15
2	b)	8	<10	5/ 6
1	b)	8	50	0/ 3
7	b)	12	10	0/21
1	b)	12	10	4/ 5*
4	b)	12	50	0/24
5 rabbits	)	40	1250	0/50
2	)	55	1250	0/20
5	)	10	6250	34/50*
2	d)	60	1250	0/10

) Attenuated Strain 119 b) Virulent Strain 119 + high molecular weight fraction of *Toxoplasma* extract. c) Attenuated Strain 119 followed by virulent Strain 119 a year later d) Virulent Strain 119 + sulfadiazine treatment.

Surviving mice had reciprocal dye test titre <10.

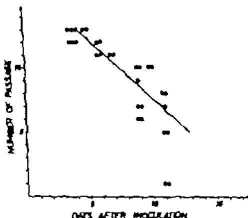


Fig 1 Increasing ·irulence of *Toxoplasma gondii* Strain 119 O = death of one mouse.

parasites were stored in undiluted exudate at 4 C for 24 hours before they were counted and inoculated into normal mice The curves A, B, C, D and 7 in Fig 2 illustrate the generation in the mouse peritoneal cavity after inoculation of 200,000, 20,000, 2,000 200 and 20 endozoites respectively Each point is the number of endozoites per mouse as calculated from aspirated fluids from 25 peritoneal cavities.

In Fig. 3 the generation of virulent Strain 119 *Toxoplasma* is compared with that of avirulent Strains 119 and 178. The endozoites and cystozoites in this experiment were count

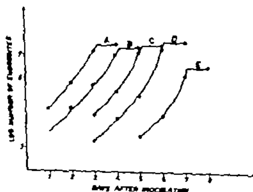


Fig. 2 Multiplication of *Toxoplasma gondii* in mouse peritoneal cavities after inoculation with decreasing numbers of ·irulent Strain 119 endozoites A 200,000. B 20,000. C 2,000 D 200. E 20 (Each point derived from 25 peritoneal fluids)

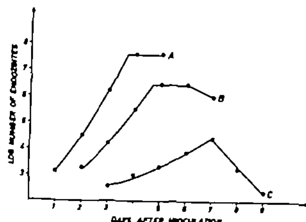


Fig 3 Multiplication of *Toxoplasma gondii* in *mouse peritoneal cavities after inoculation* of 20 000 parasites A virulent Strain 119 endozoites. B avirulent 119 strain cystozoites. C avirulent Strain 178 cystozoites. (Each point based upon 10 *peritoneal fluids)*

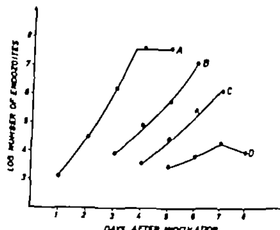


Fig 4 Multiplication of *Toxoplasma gondii* in mouse peritoneal cavities after inoculation of 20 000 virulent Strain 119 endozoites A: Immediately B After 16 hours at 37 C. C After 24 hours at 37 C. D After 40 hours at 37 C. (Each point derived from 10 peritoneal fluids)

ed and inoculated 2–3 hours after harvested from infected mice. The cystozoites were counted after pepsinizing homogenized mouse brains as previously described for rabbit brains. *Each mouse was inoculated with* approximately 2,000 parasites. Each point in the figure symbolizes the number of endozoites per mouse as calculated from fluids aspirated from 10 peritoneal cavities. The virulent Strain 119 killed all mice avirulent Strain 119 killed approximately 50 per cent, and avirulent Strain 178 killed none during the period observed After three months mice surviving inoculation with cystozoites were found to *have Toxoplasma* cysts *in their brains.*

In order to investigate the fate of parasites immediately after inoculation 200 000 virulent Strain 119 endozoites were two hours after harvested inoculated into each of 100 mice which were sacrificed in groups of ten After 50 minutes the number of parasites was 2 800 per mouse, 150 minutes after the inoculation no parasites could be detected at all whereas six hours after the inoculation the *parasites had reappeared and were calculated* to be 1 400 per peritoneal cavity From this point onwards an exponential growth curve such as A in Fig 3 and 4 was observed

The effect of storage on the multiplication rate of *Toxoplasma gondii* is illustrated in Fig 4 Curve A represents the multiplication of fresh virulent Strain 119 and curves B, C, and D similar parasites after storage at 37° C for 16 24 and 40 hours respectively in exudate diluted with one volume of phosphate buffer Each curve was derived from the inoculation of approximately 20 000 parasites, and each point in the figure symbolizes the number of endozoites per mouse as calculated from the fluids aspirated from 10 peritoneal cavities. All mice died except those inoculated after 40 hours at 37° C. Most of the surviving mice had positive dye test titres. No cysts were observed in any of these mice, although viable parasites were by *subinoculating normal mice* found to be present seven weeks after inoculation (Table 1)

The effect of storage on the virulence of *Toxoplasma gondii* is also shown in Table 2 giving the results of inoculation of virulent RH and 119 into mice before and after storage of the parasites in undiluted exudate at 37 C Three months after inoculation the brains of surviving mice with positive dye test *titres were examined microscopically* No cysts were detected Brain tissues from 10 mice were inoculated into normal mice with negative results (Table 1)

Mice inoculated with virulent endozoites in suspensions containing high molecular weight

TABLE 2. *Inoculation of Mice with Virulent* Toxoplasma gondii *after Storage in Undiluted Exudate at 37° C*

Strain	Storage (days)	Lethality* (per cent)	Average day of death	Reciprocal dye test titre (number)
119	0	100	5.8	
119	1	100	8.8	
119	2	100	10 6	
119	5	0		10 (6) 50 (4)
119	7	0		<10 (10)
RH	0	100	5.0	
RH	1	100	8.5	
RH	3	0		50 (8) 250 (2)
RH	5	0		10 (10)
RH	7	0		<10 (10)

10 mice inoculated in each group.

TABLE 3. *Inoculation of Rabbits with Virulent* Toxoplasma gondii

Strain	Attenuation	Inoculum (parasites per ml)	Died/Inoculated	Reciprocal dye test titres (number)
119	none	250	3/3	
119	none	10	1/3	10–50 (4)
119	)	250	0/6	10–50 (3) 6250 (3)
119	)	250	2/4	6250 (2)
119	b)	250	3/4	6250 (1)
RH	none	100	3/3	
RH	none	10	1/3	10–50 (2)
RH	none	1	0/3	10–50 (3)
RH	b)	150	3/4	6250 (1)

) Stored as undiluted exudate for 5 hours at 37° C b) Endozoites from mouse peritoneal fluid 7 days after inoculation with attenuated Strain 119 (Curve D Fig. 4)
Reciprocal dye test titres 10–50.

substance sometimes survived with reciprocal dye test titres of 10–50 These mice were examined for cyst formation with negative results, although (Table 1) it was possible to isolate viable parasites from them 12 weeks after inoculation.

The two virulent Strains RH and 119 were equally fatal to rabbits and mice. Rabbits surviving inoculation with these parasites had unchanged dye test titres. Some rabbits survived the inoculation with attenuated 119 with a hundred fold elevation of the reciprocal dye test titre (Table 3) These rabbits were examined for cysts with negative results. As shown in Table 4 some of these rabbits were inoculated a year later with virulent Strain 119 They survived this challenge like many of the other rabbits protected by various means against infection (Table 4) The brains from most of these rabbits were examined microscopically 2–12 months after inoculation. None was found to harbour cysts, although it was possible to isolate viable parasites from five rabbits two months after inoculation (Table 1)

In comparison three rabbits surviving inoculation with attenuated Strain 119 were a year later challenged with avirulent Strain 119 Examining the brains of these rabbits three months later cysts were found microscopically in all of them.

From Table 3 it is seen that only two out

TABLE 4 *Inoculation of Protected Rabbits with Virulent* Toxoplasma gondii

Strain	Protection	Died/Inoculated	Reciprocal dye test titres	
			Before	After (number)
119	a)	5/8	10–50	6250 (3)
119	b)	0/6	10–50	6250 (4) 10–50 (*)
119	c)	0/10	1250–6250	
119	d)	0/3	1250	
RH	a)	5/8	10–50	6250 (3)
RH	d)	0/3	6250	
RH	e)	0/3	1250	
RH	e)	2/2	250	

a) 0 1 per cent of sodium sulfadiazine in the drinking water for 18 days. b) 20 drops of Madribon on minced turnips every day for 18 days. c) Immunized with attenuated Strain 119 d) Immunized with avirulent Strain 119 e) Immunized with *Toxoplasma* antigens soluble in phosphate buffer
* Contains 20 per cent sulfadimethoxinum (F Hoffmann-La Roche et Co. Ltd.)

of 11 rabbits suffered toxoplasmosis when inoculated with a suspension containing 10 or less parasites per μl With a hundred or more parasites per μl all of the rabbits were infected The rabbits listed in Table 4 were for this reason inoculated with suspensions containing at least 250 parasites per μl

DISCUSSION

Increasing the virulence of avirulent 119 as illustrated in Fig 1 did not pose any problems other than inability to estimate the number of parasites inoculated in early blind passages with liver and spleen Eventually these parasites became as virulent for mice and rabbits as the RH strain, killing all inoculated animals.

A reciprocal dye test titre of 10–50 after incubation of sera for 30 minutes at 56 C demonstrated in most of the normal rabbits in this laboratory never posed a problem as the animals surviving experimental toxoplasmosis always displayed reciprocal dye test titres of 6250 or higher As shown in Table 4 rabbits with antibody levels less than 1250 in the dye test were not protected against a challenge with virulent *Toxoplasma* so the conceivable protection present in the normal rabbits would be negligible, and was, in fact never noticed

Investigation of parasite multiplication in the mouse peritoneum showed that the parasites disappeared within 90 minutes after inoculation. Some of the parasites presumably disseminated in the mouse causing a generalized infection (*Jacobs & Jones* 1950), and some entered host cells (*Jones et al.* 1972) making the local concentration of extracellular parasites too small for detection Six hours after the inoculation almost three per cent of the number inoculated were detected probably due to the appearance of a new generation of parasites. From this stage onwards an exponential growth of the parasite population could be followed.

By decreasing the number of parasites inoculated the corresponding generation curves appearing in Fig 2 showed the same exponential growth provided a delay proportional to the dilution of the inoculum was allowed for The sudden turn of events illustrated by the figure at a population level of approximately 40 million parasites, was not understood although it is conceivable that the physiochemical properties of the exudate itself at this stage may be an obstacle to parasitic growth. Another turn of events illustrated in Figs. 2 3 and 4 taking place around the seventh day after inoculation may possibly be correlated to a specific resistance in the mice. As would be expected from the reported variation of multiplication rate with virulence by *Kaufman et al* (1958) the slope of the curves in Fig 3 is increasing with the

virulence of inoculated parasites. From the average slope can be calculated a generation time of 4.8 hours for the virulent 119 which is in accord with that reported for the RH strain by *Kaufman & Maloney* (1962). Similarly a generation time of 6.7 hours was found for the avirulent 119 and 15 hours for the avirulent 178.

In contrast to the augmentation of avirulent 119 the attenuation of virulent 119 presented a problem due to the nature of the process itself decreasing the rate of multiplication and hence the number of parasites available for investigation. As shown in Table 2 the process of attenuation was, like that of augmentation, a gradual one. An extended storage period before inoculation of the parasites made the mice live longer until they finally survived the infection, which, according to the positive dye test titres demonstrated in these mice had taken place. The infection was furthermore proved by curve D in Fig. 4 which illustrates the generation of the attenuated parasites used for the inoculation of some of these mice surviving with a positive dye test titre. When the storage period was extended still further the parasites were obviously not viable any longer as the mice survived with a reciprocal dye test titre <10.

The curves in Fig. 4 suggest a closer relationship of the attenuated parasites to the avirulent than to the virulent ones. It was, however, found that subinoculation of endozoites from mice seven days after inoculation with attenuated 119 gave four days later peritoneal exudates containing averagely 25 per cent of the number of endozoites expected from the results of inoculation with virulent 119. This was a very high yield compared to the generation curve of attenuated parasites. The main effect of the attenuation was, therefore, considered to be on the parental generation. On the other hand as long as the yield was less than with the virulent 119 this attenuation process may have some genetic effect. This conjecture was substantiated by the survival of two out of eight rabbits inoculated with endozoites isolated from mice inoculated with attenuated 119. Compared to no survivors after inoculation of ten rabbits with virulent 119 this result (Table 3) is highly significant.

In accord with the results reported by *Frenkel* (1954), *Beverley & Fry* (1957), *Midtvedt* (1964) and *Lan et al.* (1974) it was, as shown in Table 1, possible to isolate virulent parasites from mice and rabbits up to three months after an inoculation with virulent parasites. Contrary to *Nakayama & Matsubayashi* (1961) no cysts were ever detected by direct microscopy of brain smears from such animals. Furthermore isolation experiments from mice and rabbits with a history comparable to those mentioned above were following this three months period without demonstrable parasites as shown in Table 1. It thus appeared that the ability of *Toxoplasma gondii* to form cysts in mice and rabbits was lost at some point during the augmentation of virulence. The virulent parasites present in these animals were more vulnerable presumably to specific immunity than encysted parasites.

I am grateful to Mrs. *E. Matjeka* and Mr. *K. Glad* for help in this work.

In part aided by grant to the Department of Toxoplasmosis and Viral Diseases from *US Public Health Service Grant Foundation* (AI 1741), Bethesda, Md. USA.

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K ANTIGEN DETERMINATION OF *ESCHERICHIA COLI* BY COUNTER-CURRENT IMMUNOELECTROPHORESIS (CIE)

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Semjén, G., Ørskov I. & Ørskov F. K antigen determination of *Escherichia coli* by counter current immunoelectrophoresis (CIE). Acta path. microbiol. scand. Sect. B, 85 103–107 1977

The application of the counter-current immunoelectrophoresis (CIE) for determination of *E. coli* acidic polysaccharide K antigen is described. The most appropriate dilutions of antigens and antisera were established after examination of six different K antigens with homologous OK antisera. According to this result all test strains for acidic polysaccharide K antigens, i.e. K1 to K57 K62, K74 K82, K83, K84 K92, K93 K94 K95 K96, K97 K98 and K100, were examined and OK antisera suitable for the CIE test were selected. *The following* reciprocal cross-reactions were found: K2 and K62 K7 and K56 K12 and K82 K13 K20 and K23; K18 and K22 K16 and K97 and K35 and K93 The *CIE* method is now used as routine test in our laboratory

Key words *Escherichia coli*; K antigen determination counter-current immunoelectrophoresis.

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Received 21. 76 Accepted 21.x.76

The established use of bacterial agglutination technique has been questioned in several recent papers by *Ørskov et al* when used as the only technique for determination of *Escherichia coli* K antigens (8, 10, 13 14) *In the agglutination test live bacteria are ex*amined—on slide or in tube—in appropriate OK antisera. The resulting agglutination will often be the composite reaction of several surface antigens and their corresponding antibodies. Some such reactions will be caused by the specific K antigens others will be determined by additional surface antigens many of which are common to many strains. Some can be caused by H antigens.

In contrast, a gel precipitation technique gives a neat separation of the polysaccharide antigens of *E. coli* and particularly immunoelectrophoresis has been used for a more precise delimitation of these antigens (8, 9 10 11 13, 14) For practical reasons it will be useful at this juncture to describe the following categories of K antigens

E. coli acidic polysaccharide K antigens found in connection with O8 O9 O20 and O101 probably constitute a special category since those examined are, to some extent at least, covalently linked to the core-lipid A structure of the cell wall and can be con-

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sidered as K lipopolysaccharide (LPS) Thus, such strains may contain both O LPS *e g* O8 and K LPS *e g* K25 (15) These K antigens (in O8 O9 O20 and O101) are genetically determined by a chromosomal locus (or loci) different from that of polysaccharide K antigens found in connection with all other O antigens (7) (see below)

Acidic polysaccharide K antigens in strains with other O groups than O8 O9 O20 and O101 These are the K antigens which can easily be separated from the O antigens and which are determined genetically by a locus called *Lps A* (12 16)

Protein K antigens The so far established ones K88 and K99 are filamentous and determined by plasmids. Other antigens (4) probably belong to this category The typing of these antigens, which are developed at 37 C and not at 18° C is usually carried out by slide agglutination of cultures grown at both temperatures. As these antigens are truly thermolabile being destroyed by heating at 100° C, pure K sera can be prepared by absorption of OK antisera with boiled culture

The acidic polysaccharide K antigens, however are often difficult to type by slide agglutination because of the above mentioned problems. We have therefore tried to adapt counter-current immunoelectrophoresis (CIE)—successfully used in connection with other acidic capsular polysaccharides (2 3 5) —for K determination of *E coli*

For a further review of the *E coli* K antigens, see Ref 15

MATERIALS AND METHODS

Strains E. coli antigen test strains for acidic polysaccharide K antigens, i e K1 to K57 and furthermore K62 K74 K82 K83 K84 K87 K92 K93 K94 K95 K96 K97 K98 and K100 were included A number of *E coli strains received in* our laboratory originating from different sources, were also examined

Extract production (antigens) A simple saline extract from agar plate cultures (about 5 × 10[1] bacteria per ml) was prepared as described earlier (11) Recently an even more simple antigen preparation has been used, *i.e* the bacterial suspension is heated at 100 C directly and not centrifuged afterwards

Antisera Rabbit O and OK sera, most of them routine sera from our laboratory were used. Dilution of antisera and extracts were made in veronal buffer (see below)

Counter-current immunoelectrophoresis (CIE) was performed in electrophoresis equipment from Dansk Laboratoriesudstyr (Copenhagen) Glass plates (10 × 10 cm) were covered with 15 ml 1 per cent agarose (Litex Copenhagen) in veronal buffer pH 8 6 ionic strength 0 05 μ. Three sets of double rows each containing nine wells (4 mm diameter) 54 in all, were cut in the agar The distance from upper to lower well in a double row was 4 mm and between two adjacent holes 5.5 mm 10 μl (we now use 20 μl) of antigen was placed into the wells of one row and those of the other row were filled with the same amount of antiserum. Antigen was used in different dilutions, while antisera were generally diluted 1·4 or undiluted The electrophoretic run was performed at 2.5 V per cm for 1 hour at room temperature with the antiserum wells on the anodic side Watman's filter paper No. 7 was used as wicks. The buffer was veronal buffer pH 8.6 ionic strength 0.05 μ After cooling for 5–10 minutes the slides were examined for precipitation lines and results were recorded by drawing

RESULTS

Selection of Optimal Concentrations of Antiserum and Antigen

In order to avoid interference of thermolabile substances only 100 C extracts were used as antigen Six strains with known acidic polysaccharide K antigens were examined against homologous undiluted OK sera. Two antisera from different rabbits were used for each extract. Undiluted and two-fold dilutions up to 1 256 of extracts were employed. All dilutions gave precipitation lines, but the undiluted and the lower dilutions of antigen gave poorly defined blurred lines close to the serum well. Optimal precipitation was generally obtained with dilutions 1 100 and 1.200 By use of these antigen dilutions some antisera could be employed in dilution 1 32. However dilution 1·4 was ordinarily considered most appropriate.

Examination of the E coli K antigen Test Strains

100 C extracts of the above listed 71 *E. coli* test strains with acidic polysaccharide K anti

gens were examined against the corresponding OK antisera. OK sera diluted 1:4 and undiluted were used, while antigens were employed as 1:50 and 1:200 dilutions of the 100 C extracts. In all cases, except K1 and K5 (see below) precipitation lines were found. The sera which only reacted undiluted were regarded as useless for this test and, if required, new were prepared. O sera against strains with non-acidic LPS O antigens showed no precipitation. However with four strains, O83:K24 O139:K82, O150:K93 and O81:K97 double lines were detected these strains are K test strains that, in addition to an acidic K polysaccharide, produce an anodic LPS line. Only strains with such an acidic LPS will show a precipitation line in O antisera. It could also be mentioned here that only the expected K line—and no O line—appeared when strains with the same O but different K antigen were examined, e.g. O4 strains with K3 K6, K12 or K52 O6 with K12, K15 K53 and K54 O20 with K17 K83 and K84 and O8 and O9 strains with many different K antigens These results support the reliability of the CIE test for determination of acidic polysaccharide antigens.

However in the cases of K1 and K5 the CIE test could not be used. We have no more K5 antiserum and have not succeeded hitherto in preparing a new antiserum in rabbits, although we know from the test where agar electrophoresis is combined with Cetavlon precipitation in second dimension (6) that the K5 test strain still contains an acidic polysaccharide. An antiserum against K1 is not impossible to obtain, but it is very difficult to prepare in rabbits. To-day we take advantage of the identity between *E. coli* K1 and the capsular substance of *Neisseria meningitidis* type B and use a horse antiserum (generously provided by *John B. Robbins* N.I.H. Washington D.C.) against the latter organism for determination of K1 in *E. coli* (17). This antiserum can easily be used in CIE.

Preparation of K Serum Pools

In order to simplify the examination of unknown strains the K test sera were pooled. Each pool contained four OK sera, thus making the dilution of the single serum 1:4. In this series the pools were composed without consideration of the cross-reactions between K antigens it would, however be preferable to pool cross-reacting sera.

From earlier investigations of cross-reactions between the K antigens, carried out by slide agglutination and double diffusion in gel, it was known that some K strains cross-reacted in heterologous OK sera. In order to examine how many of such reactions were due to significant cross-reactions between the polysaccharide K antigens, extracts and sera were examined in CIE. 100 C extracts, diluted 1:200 of the cross-reacting strains, i.e. K2, K3 K4 K6, K7 K11 K12, K13 K16 K18, K19 K20 K21 K22, K23 K24 K26, K37 K43 K45 K47 K49 K50, K52 K53 K54 K56 K62, K74 K82, K93 K95 K96, K97 K98 and K100, were examined against OK sera to all acidic polysaccharide K antigens.

TABLE 1 *Cross-Reactions of* E. coli *Acidic Polysaccharide K Antigens Demonstrated by CIE*

OK antisera	Reactions with K test strains
K2	K2, K62
K62	K62, K2
K7	K7 K56
K56	K56, K7
K12	K12, K82
K82	K82, K12
K13	K13 K20 K23
K20	K20 K13 K23
K23	K23 K13 K20
K16	K16, K97
K97	K97 K16, K37
K18	K18, K22
K22	K22, K18
K53	K53, K93
K93	K93, K53

Table 1 shows the demonstrated cross-reactions, all of which were reciprocal except K37 and K97 Further studies have shown that K2 is so closely related to K62, K7 to K56 and K12 to K82 that K62, K56 and K82 should be deleted as K antigens in favour of K2, K7 and K12, respectively (15) The

sidered as K lipopolysaccharide (LPS) Thus, such strains may contain both O LPS *e g* O8 and K LPS *e g* K25 (15) These K antigens (in O8 O9 O20 and O101) are genetically determined by a chromosomal locus (or loci) different from that of polysaccharide K antigens found in connection with all other O antigens (7) (see below)

Acidic polysaccharide K antigens in strains with other O groups than O8 O9 O20 and O101 These are the K antigens which can easily be separated from the O antigens and which are determined genetically by a locus called *kps A* (12 16)

Protein K antigens The so far established ones, K88 and K99 are filamentous and determined by plasmids. Other antigens (4) probably belong to this category The typing of these antigens, which are developed at 37 C and not at 18° C is usually carried out by slide agglutination of cultures grown at both temperatures. As these antigens are truly thermolabile being destroyed by heating at 100° C pure K sera can be prepared by absorption of OK antisera with boiled culture

The acidic polysaccharide K antigens, however are often difficult to type by slide agglutination because of the above mentioned problems. We have therefore tried to adapt counter-current immunoelectrophoresis (CIE)—successfully used in connection with other acidic capsular polysaccharides (2 3 5) —for K determination of *E coli*

For a further review of the *E coli* K antigens, see Ref 15

MATERIALS AND METHODS

Strains *E coli* antigen test strains for acidic polysaccharide K antigens, i.e. K1 to K57 and furthermore K62 K74 K8? K83 K84 K87 K92, K93 K94 K95 K96 K97 K98 and K100 were included A number of *E coli* strains received in our laboratory originating from different sources, were also examined.

Extract production (antigens) A simple saline extract from agar plate cultures (about 5×10^{10} bacteria per ml) was prepared as described earlier (11) Recently an even more simple antigen preparation has been used, *i.e.* the bacterial suspension is heated at 100 C directly and not centrifuged afterwards.

Antisera Rabbit O and OK sera, most of them routine sera from our laboratory were used. Dilution of antisera and extracts were made in veronal buffer (see below)

Counter-current immunoelectrophoresis (CIE) was performed in electrophoresis equipment from Dansk Laboratorieudstyr (Copenhagen) Glass plates (10 × 10 cm) were covered with 15 ml 1 per cent agarose (Litex, Copenhagen) in veronal buffer pH 8 6 ionic strength 0.05 µ. Three sets of double rows each containing nine wells (4 mm diameter) 54 in all were cut in the agar. The distance from upper to lower well in a double row was 4 mm and between two adjacent holes 5.5 mm 10 µl (we now use 20 µl) of antigen was placed into the wells of one row and those of the other row were filled with the same amount of antiserum. Antigen was used in different dilutions, while antisera were generally diluted 1·4 or undiluted The electrophoretic run was performed at 2.5 V per cm for 1 hour at room temperature with the antiserum wells on the anodic side. Watman's filter paper No. 7 was used as wicks. The buffer was veronal buffer pH 8 6 ionic strength 0.05 µ After cooling for 5–10 minutes the slides were examined for precipitation lines and results were recorded by drawing

RESULTS

Selection of Optimal Concentrations of Antiserum and Antigen

In order to avoid interference of thermolabile substances only 100° C extracts were used as antigen. Six strains with known acidic polysaccharide K antigens were examined against homologous undiluted OK sera. Two antisera from different rabbits were used for each extract. Undiluted and two-fold dilutions up to 1 256 of extracts were employed. All dilutions gave precipitation lines, but the undiluted and the lower dilutions of antigen gave poorly defined blurred lines close to the serum well Optimal precipitation was generally obtained with dilutions 1 100 and 1 200. By use of these antigen dilutions some antisera could be employed in dilution 1 32. However dilution 1 4 was ordinarily considered most appropriate.

Examination of the E. coli *K antigen Test Strains*

100 C extracts of the above listed 71 *E. coli* test strains with acidic polysaccharide K anti

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other cross-reactions mentioned need further examination therefore more K antigens may be deleted later

Since the introduction in our laboratory of the CIE method a number of unknown strains have been examined for their K antigens. 100° C extracts, diluted 1 200 have been examined in the pools and if positive reactions occurred tests were made in the single sera undiluted or diluted 1 4 Results have often been further confirmed by ordinary immunoelectrophoresis.

DISCUSSION

The CIE test which has been found useful for determination of several bacterial acidic polysaccharide capsule antigens, is highly applicable to *E coli* acidic polysaccharide K antigens and it may also be useful in connection with such antigen from other *Enterobacteriaceae* e g *Klebsiella* However its specific value for typing of *E coli* K antigens is caused by the fact that other K detection methods like slide agglutination and capsule quellung test may give results which are not simple to evaluate Slide agglutination, which is an extremely easy test for K antigen screening will often show too many cross reactions due to interference of other surface antigens and the capsule quellung test will seldom be strong enough because of the comparatively small capsules of *E coli* As pointed out several times in connection with other examinations of *E coli* K antigens, ordinary immunoelectrophoresis is a good method for demonstration of a suspected acidic polysaccharide K antigen particularly when the strain agglutinates in more than one OK antiserum Satisfactory K antigen determinations have hitherto only been carried out to a very limited extent even in specialized laboratories. However the CIE described here is so reliable that determinations of these K antigens could be a common procedure in such laboratories. Because of the large number of antisera required we do not expect that such K determinations will be in widespread use in non specialized laboratories.

However the method is also applicable in cases where only a few acidic polysaccharide K antigens are searched for among many coli strains, i.e when extracts have to be tested against one or a few sera yet it should be remembered that the antiserum plate method (1) can be very useful for this purpose. The CIE technique has now been used in our laboratory for some time and as expected, many strains do not react in the test. In order to find out whether such negative strains have a polysaccharide K antigen one has to show the existence of an acidic polysaccharide substance. The classical O inagglutinability test, i.e a negative agglutination test of the living strain in homologous O serum is a strong but not always satisfactory indication of presence of a polysaccharide K antigen, and particularly with O agglutinable strains one is left in doubt as to the presence of such an antigen Recently we have developed a test, combining agarose electrophoresis with Cetavlon precipitation in second dimension, which demonstrates unequivocally the existence of an acidic polysaccharide (6) If further analysis of the K antigen is needed when this test is positive, an OK serum is produced and cross-examinations against all established K antigens and K antisera are carried out with the CIE. Finally the new K serum receives its place in a K serum pool. Many new K antigens will probably have to be established before we shall be able to determine a reasonably high percentage of acidic polysaccharide K antigens in *E coli* strains.

We thank *Betty Wolff* for excellent technical assistance

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Discussion

Suitable experimental animals are not always at hand for assisting the isolation of fastidious organisms, and even when such animals are at disposal, contaminating flora may be pathogenic for the animal. Considering the cultivation of *F tularensis* the easily available, selective CLNT-medium could be practical supplement to the non-selective ones, especially useful when animal inoculation is impracticable. Although the possibility exists that our strains might be better adapted to artificial conditions than wild strains, passage in mice indicated that successful cultivation was not related to virulence.

We consider the inclusion of trimethoprim in the medium particularly important, because it is highly desirable to inhibit the swarming of *Proteus* sp commonly encountered in samples.

With the widespread distribution of *F tularensis* and the very low frequency of bacterial isolation attained (6) we suggest that the described selective cultivation method may be useful improvement.

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BRIEF REPORTS

CULTIVATION AND ISOLATION OF *FRANCISELLA TULARENSIS* ON SELECTIVE CHOCOLATE AGAR, AS USED ROUTINELY FOR THE ISOLATION OF GONOCOCCI

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Berdal B P & Soderlund, E. Cultivation and isolation of *Francisella tularensis* on selective chocolate agar as used routinely for the isolation of gonococci. Acta path. microbiol. scand. Sect. B, 85 108–109 1977

Two strains of *Francisella tularensis* were grown on a selective chocolate agar used for the isolation of gonococci. The growth conditions appeared to be satisfactory and contaminating flora was largely eliminated thus facilitating reisolation and diagnosis.

Key words F *tularensis* selective medium.

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Received 26.xi 76 Accepted 9.xii 76

F tularensis is considered as a fastidious organism growing only on rich media, of which the most commonly used is glucose-blood-cysteine agar (GBC-agar) with the addition of rabbit or horse blood (1 2 4) For primary isolation, intraperitoneal inoculation of the sample material into a suitable animal (mouse or guinea pig) is often a necessary step enriching and simplifying the flora before growth on a solid medium The increased use of selective growth media in routine gonococcal diagnosis, induced us to investigate the practical use of these media for the isolation of *F tularensis* based on the similarity we have observed in antibiotic sensitivity between *Neisseria gonorrhoea* and *F tularensis*

Methods and results

1) Two trains of *F tularensis* Vax (originating from the attenuated live strain LVS developed as a live bacterial vaccine) (3) and *Hare* (HN 63) (5) were cultivated on GBC-agar chocolate agar and the Thayer Martin derivative CLNT agar (chocolate agar with colistin 7.5 µg/ml nystatin 12.5 µg/ml, lincomycin 0.5 µg/ml and trimethoprim lactate 5 µg/ml added) (7 8) Incubation at 37°C in an athmosphere containing 5 per cent CO_2 for 2–3 days produced smooth colonies of 0 5–1 mm diameter the growth on the non-selective medium, however being somewhat livelier than that on the selective medium. Stable growth was observed after repeated passage on these media (10 times)

2) The Vax strain was introduced into 10 mixed flora from human throat sub-cultures *Streptococcus* sp., *Micrococcus* sp., and Diphteroids, were predominant in the mixture

The Vax strain could be reisolated from 6 of the 10 mixtures.

3) White mice (NMRI/Bom) were inoculated intraperitoneally with 0 5 ml of a heavy suspension (containing at least 10 million viable bacteria per ml) of Vax strain or *Hare* strain These inoculations are usually lethal within 4 days. Cultures prepared from pairs of mice one to three days after inoculation (sacrified or diseased animals) on chocolate agar and CLNT-agar showed rich and sometimes heavily contaminated growth on the chocolate agar whereas the CLNT-agar showed *F tularensis* either in pure culture or only slightly contaminated.

Discussion

Suitable experimental animals are not always at hand for assisting the isolation of fastidious organisms, and even when such animals are at disposal, contaminating flora may be pathogenic for the animal. Considering the cultivation of *F tularensis* the easily available, selective CLNT-medium could be a practical supplement to the non-selective ones, especially useful when animal inoculation is impracticable. Although the possibility exists that our strains might be better adapted to artificial conditions than wild strains, passage in mice indicated that successful cultivation was not related to virulence.

We consider the inclusion of trimethoprim in the medium particularly important, because it is highly desirable to inhibit the swarming of *Proteus* sp., commonly encountered in samples.

With the widespread distribution of *F tularensis* and the very low frequency of bacterial isolation attained (6) we suggest that the described selective cultivation method may be a useful improvement.

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Acta path. microbiol. scand Sect. B, 85 110–112 1977

OBSERVATIONS ON THE ULTRASTRUCTURE OF THE LATE SPOROBLAST AND THE INITIATION OF SPOROZOITE FORMATION IN *EIMERIA BRUNETTI*

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Ferguson D J P., Birch Andersen A., Hutchison, W M & Siim, J Chr Observations on the ultrastructure of the late sporoblast and the initiation of sporozoite formation in *Eimeria brunetti*. Acta path microbiol. scand Sect. B, 85 110–112 1977

The ultrastructure of the late sporoblast and the initiation of sporozoite formation in *E. brunetti* was examined in oocysts which had been allowed to sporulate for 24 hours at 27 C The late sporoblast was ellipsoidal in shape and possessed two nuclei, with associated Golgi bodies, situated at either end of the organism. Its cytoplasm contained numerous polysaccharide granules, electron translucent vacuoles, dense bodies, mitochondria, and some strands of rough endoplasmic reticulum. The sporoblast at this stage was enclosed by two unit membranes. Sporozoite formation was initiated by the appearance of a dense plaque at either end of the organism in the vicinity of a nucleus, adjacent to the limiting membrane of the sporoblast. A conoid was present in the central region of the plaques. Sporozoite formation was similar to the initiation of merozoite formation in the schizont. Endodyogeny did not appear to be involved in the process of sporozoite formation

Key words *Eimeria brunetti* late sporoblast initiation of sporozoite formation ultrastructure.

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Received 18.x 76 Accepted 17.xii.76

Parasites belonging to the family Eimeriidae of the coccidia produce oocysts which develop (sporulate) in the external environment to produce sporozoites which are the infective forms The oocyst walls are resistant to chemical penetration and consequently the ultrastructural aspects of this sporulation have not been described because of difficulties in preparing the oocysts for ultrastructural examination Recently however a method for preparing thin sections of coccidian oocysts for electron microscopy has been described (1) In this note will be reported some results obtained with this technique on the late sporoblast and the initiation of sporozoite formation in *Eimeria brunetti* a coccidian parasite of domestic fowls.

Materials and Methods

Oocysts, which had been concentrated from chicken faeces, were allowed to sporulate for 24 hours at 27 C before being processed for electron microscopy The technique used can be summarised as follows (for details see 1) Oocysts were pre-embedded in cross linked bovine serum albumin and sectioned with a cryostat after rapid freezing in liquid nitrogen. The cryostat sections were allowed to thaw in Karnovsky's fixative (3) then

* Work initiated while a Wellcome Trust Travelling Research Fellow and completed as a Danish Medical Research Council Fellow

** Aided by grants from the *World Health Organization* Geneva *The Wellcome Trust* and the *British Council*



Figures 1-4 are micrographs of sporoblasts of *E. brunetti* observed within oocysts which had been allowed to sporulate for 24 hours at 27 C. A single bar ( ) on micrograph represents 100 nm and double bar ( ) represents 1 µm.

postfixed in osmium tetroxide and finally embedded in Vestopal-W. Thin sections were examined after staining with magnesium uranyl acetate and lead citrate.

Results

In the material thus prepared it was possible to find sections of oocysts which had reached the sporoblast stage of development. We use the term late sporoblast because although the Stieda body has started to develop the sporocyst wall is still unformed. The late sporoblasts were limited by two unit membranes (Fig. 2) and it was possible to observe micropores associated with these membranes (Fig. 1). At this stage, the organism was ellipsoidal in shape and the initial formation of a Stieda body at one end of the sporoblast could be observed (Fig. 1 small arrow). In addition, the developing organism at this stage, possessed two nuclei which were situated at opposite ends of the sporoblast (Fig. 1). Golgi bodies were found adjacent to the nuclei (Figs. 1 & 3) and the cytoplasm of the organism also contained a number of polysaccharide granules, electron translucent vacuoles, dense bodies of amorphous material, mitochondria, and some strands of rough endoplasmic reticulum (Fig. 1).

The first sign of sporozoite formation was indicated by the appearance of a dense plaque at either end of the organism in the region of a nucleus and adjacent to the two limiting membranes of the organism (Figs. 1 & 4). A conoid was observed in the central part of these plaques and it was possible to distinguish unit membranes within the dense material of the plaques (Fig. 4).

Discussion

Ultrastructural details of the late sporoblasts of other *Eimeria spp.* have not been described, but one of the characteristics described here for *E. brunetti* resembles one described for the sporocysts of *Sarcocystis tenella* (4), i.e. the observation of a nucleus positioned at either end of the organism. On the other hand, the late sporoblasts of *E. brunetti* differ from those of *S. tenella* (4) in possessing electron translucent vacuoles and dense bodies and by lacking lipid globules.

In the present study the first evidence of sporozoite formation was the presence of a dense plaque with associated conoid at the limiting membranes of the organism in the vicinity of a nucleus. This observation is similar to that described for the initiation of merozoite formation in the schizonts of *E. brunetti* observed within the epithelial cells of infected chickens (2). From studies on schizogony of the coccidia it has been proposed that endodyogeny* is the fundamental process of asexual multiplication in this group (5). Accordingly it would be expected that sporozoite formation would also occur by endodyogeny but, from our observations, this does not appear to be the case. In *E. brunetti* as in other *Eimeria spp.*, only two sporozoites are found within each sporocyst. This is in contrast to *Isospora spp.* and *Sarcocystis spp.* where four sporozoites are formed in each sporocyst. Thus, the finding of a nucleus at either end of the organism at the initiation of sporozoite formation would indicate that the final nuclear division already had occurred and that only one sporozoite will be formed in association with each of the two nuclei.

Fig. 1. A longitudinal section through a sporoblast in which an incompletely formed Stieda body is present at one end (small arrow) and a micropore (MP) is present at the limiting membranes. The cytoplasm contains a nucleus (N) with associated Golgi body (G) at either end of the organism as well as a number of polysaccharide granules (PG), dense bodies (D), mitochondria (MI), electron translucent vacuoles (EV), and a few strands of the rough endoplasmic reticulum (ER). Dense plaques are present at either end of the organism (large arrows). × 15,000.

Fig. 2. An enlargement of part of the periphery of the sporoblast in Fig. 1. Note that the sporoblast is limited by two unit membranes (arrows). × 90 000.

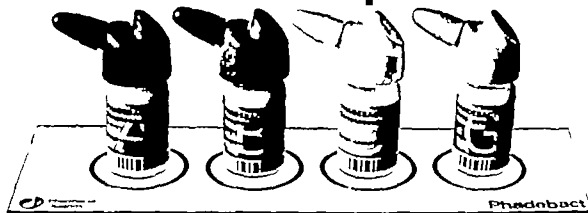
Fig. 3. An enlargement of part of the cytoplasm of a sporoblast in which a nucleus (N), a Golgi body (G) and a mitochondrion (MI) can be seen. × 90 000.

Fig. 4. Part of the periphery of a sporoblast in which the dense plaque which represents the initiation of sporozoite formation is shown together with part of a nucleus (N). The plaque is adjacent to the limiting membranes (LM) of the organism and a conoid (C) is situated in its central region. Pieces of unit membranes are present within the dense material of the plaque (arrows). × 90,000.

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* Endodyogeny is the term used to describe a process of asexual multiplication in which daughter formation is occurring during and associated with the final nuclear division, and thus each nucleus divides between two developing daughters (5).

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Acta path. microbiol. scand. Sect. B 85 115-116 1977

SOLID-PHASE RADIOIMMUNOASSAY DETECTION OF RUBELLA VIRUS IgG ANTIBODY IN SERUM AND CSF OF PATIENTS WITH MULTIPLE SCLEROSIS

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Meurman, O. H., Arstila, P. P., Panelius, M., Reunanen, M. I., Viljanen, M. K. & Halonen, P. E. Solid-phase radioimmunoassay detection of rubella virus IgG antibody in serum and CSF of patients with multiple sclerosis. Acta path. microbiol. scand. Sect. B 85: 115-116, 1977.

Low levels of rubella virus IgG antibody have been detected in cerebrospinal fluid (CSF) specimens of multiple sclerous (MS) patients by a solid-phase radioimmunoassay (RIA) previously developed for testing of clinical serum samples. Paired serum and CSF specimens of 36 MS patients and 12 control patients were analyzed. Of those MS patients which had negative CSF titers in the rubella hemagglutination inhibition (HAI) test, 90 per cent (11/12) were found to have rubella IgG antibody by the RIA method. In specimens found to contain rubella IgG antibodies by both methods, the RIA test was approximately 15 times more sensitive than the rubella HAI test. The results presented suggest that a considerable portion of MS patients has significantly reduced serum/CSF rubella IgG antibody ratios, indicating that there is a local production of rubella antibodies in the central nervous system of these patients.

Key words: Rubella virus; IgG antibody; radioimmunoassay; multiple sclerosis; serum; CSF.

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Received 4. 76. Accepted 4. 76

Measurement of anti-viral antibodies in serum and cerebrospinal fluid (CSF) specimens is of considerable importance when studying the possible viral etiology of certain progressive neurological disorders. From such data calculations of serum/CSF antibody titer ratios can be made and if a significantly reduced ratio can be demonstrated without coexistent damage of the blood-brain barrier, then intracerebral synthesis of antibodies against the virus in question is indicated. Such studies have been performed on serum and CSF specimens from patients with multiple sclerosis (MS) and reduced serum/CSF titer ratios have been found for antibodies directed against measles, rubella, mumps, and herpes simplex viruses (4).

The relatively low sensitivity of conventional serological techniques such as the complement fixation (CF) and hemagglutination inhibition (HAI) test is a major disadvant

age when measuring low antibody levels of CSF specimens Moreover the HAI test must be done on CSF samples without prior removal of nonspecific inhibitors since the treatment procedure involved also removes some of the specific antibodies. As a consequence the reliability of low HAI titres in CSF specimens remains questionable.

A sensitive and reliable solid phase radioimmunoassay (RIA) has recently been developed for detection of rubella virus IgG and IgM antibodies in serum specimens (2) In the present study this RIA method is tested for suitability in measuring low level rubella virus antibodies in the CSF of MS and control patients The data obtained have been used to calculate serum/CSF rubella antibody ratios. As well, the results presented allow a direct comparison to be made between the sensitivities of the rubella HAI and RIA tests.

MATERIALS AND METHODS

Serum and Cerebrospinal Fluid Specimens

Paired serum and CSF specimens from 36 MS patients and 12 control patients with other neurological or psychiatric disorders were included. The patients were selected from a larger material which are described in detail elsewhere (5) All patients had rubella HAI antibody in their sera. The MS patients were divided into three groups of 12 patients each on the basis of rubella HAI antibody titers in their CSF The HAI-titers in the first second and third groups were >2 2 and <2 respectively All control patients had a CSF rubella HAI titer of <2 The MS patients had a mean age of 36 years and comprised 12 males and 24 females. In the control group there were 3 males and 9 females with a mean age of 44 years

RIA Procedure

The methods used in this study have been described in detail earlier (2) Briefly rubella virus grown in suspension culture of *BHK 21/13S cells* was concentrated by Amicon filtration and purified by ultracentrifugation through 20 per cent sucrose. The purified rubella virus antigen was adsorbed onto polystyrene balls (6 4 mm diameter Precision Plastic Ball Co. Chicago Ill) by incubating submerged balls at room temperature overnight in an antigen solution containing approximately 110 µg protein/ml Dried balls were then incubated at 37 C for 1 hour in four fold serial dilutions of serum or CSF specimens, beginning with the dilution of 1 4 After washing the balls were incubated in a solution of ^{125}I labelled anti-human-gamma immunoglobulin at 37 C for 1 hour Following washing the balls were assayed for bound radioactivity in an LKB Wallac 1280 gammacounter The RIA antibody results are expressed as serum and CSF titers. For calculating the end-point titers only the linearly declining part of the cpm versus dilution curves was used. The cut-off point used for positive specimens was three times the average cpm of the negative control serum or CSF Using the cut-off point, the end point titer of a specimen was then read from the dilution scale as the reciprocal of the nearest two-fold serum dilution This method for determining the RIA titers was more reliable than the method described earlier The use of only the linearly declining part of the dilution curves, however did give somewhat lower serum titers than previously

HAI Tests

The tests were performed by microtechnique according to the modified rubella HAI test employed at the Center for Disease Control, Atlanta Ga (6) The CSF specimens were tested without pretreatment to remove nonspecific inhibitors.

Immunochemical Methods

The concentrations of IgG and albumin were determined in serum and CSF specimens by the single radial immunodiffusion method described by *Mancini et al.* (3)

RESULTS

The geometric means of rubella HAI and RIA titers in serum and CSF specimens are listed in Table 1 From this data it was ascertained that the RIA method is approximately 15 times more sensitive than the HAI test CSF from only one MS patient, out of 36 tested was found to be negative in the RIA test The rubella HAI titer of this particular sample was <2. In contrast, only 2 out of 12 control CSF specimens tested were found to contain rubella antibodies. For both patients, the rubella RIA titers of serum and CSF were 2048 and 8 respectively

Albumin and IgG concentrations were determined for serum and CSF specimens from 29 MS patients and from a single control patient (data not shown) As expected ele-

TABLE 1 *Geometric Means of Rubella HAI and RIA-titers in Serum and Cerebrospinal Fluid (CSF) Specimens in Three Groups of Patients with Multiple Sclerosis (MS) and in a Control Group*

Patient group	Mean HAI-titer		Mean RIA-titer	
	Serum	CSF	Serum	CSF
MS patients CSF HAI titer >2	384	8.48	5790	192
MS patients CSF HAI-titer = 2	114	2	1450	32.0
MS patients CSF HAI-titer <2	95.9	<2	1290	20.6‡
Control patients CSF HAI-titer <2	60.4	<2	683	8†

Each group contained 12 patients

‡ Calculated from 11 positive.

† Only two positive.

TABLE 2 *Arithmetic Means of Serum/ Cerebrospinal Fluid (CSF) Ratios of HAI-titers, RIA-titers IgG Concentrations and Albumin Concentrations in Three Groups of Patients with Multiple Sclerosis (MS) and in Control Group*

Patient group	Arithmetic means of serum/CSF ratios of			
	HAI titers	RIA-titers	IgG concentrations	Albumin concentrations
MS patients CSF HAI-titer >2	48	39	119	323
MS patients CSF HAI-titer 2	81	51	156	389
MS patients CSF HAI-titer <2	>75	103‡	158†	494†
Control patients CSF HAI-titer <2	>56	>225	479þ	246þ

Each group contained 12 patients.

‡ Calculated from 11 positive for both serum and CSF

† Five patients tested

þ One patient tested

ated IgG concentrations were found in the MS patient CSF specimens.

Table 2 summarizes the mean serum/CSF ratios calculated from HAI titers, RIA-titers, IgG concentrations, and albumin concentrations. In all three MS groups, there was a reduced mean serum/CSF ratio of rubella antibodies measured with both the HAI test and the RIA test. If a ratio of 64 is taken as subnormal and 128 as normal, only 7 out of 36 MS patients had a normal serum/CSF ratio based on RIA titers. Of these seven patients, six belonged to the MS group having a CSF HAI titer of <2. None of the control patients had a reduced serum/CSF ratio based on RIA titers. For the two control patients possessing CSF rubella antibodies as detected by the RIA method, the serum/CSF ratio was found to be 256. In each of the three MS patient groups, the mean serum/CSF IgG ratio was reduced when compared to controls. The reduction was significantly less, however, than that seen in the MS patient mean serum/CSF rubella antibody ratios. Since the mean serum/CSF ratios for albumin concentrations were normal in the MS patient groups, damage to the blood-brain barrier can be excluded as an explanation for the reduced serum/CSF rubella antibody ratios in these patients.

DISCUSSION

The solid-phase RIA method originally developed for detecting rubella IgG antibodies in serum specimens has been found to be suffi-

ciently sensitive for measuring low rubella IgG antibody levels in CSF specimens. The results obtained parallel those of the rubella HAI test. The higher sensitivity of the RIA method however makes it possible to detect antibody levels far below the detection limits of the HAI test. Similar results have been obtained by *Arstila et al* (1) who have used an analogous solid phase RIA for measuring measles antibodies in serum and CSF specimens.

The increase in sensitivity gained by the RIA test, compared to the HAI test was slightly greater when CSF specimens were tested instead of serum specimens. As a consequence, the mean serum/CSF rubella antibody ratios based on the RIA test results were somewhat lower than those based on the HAI test results. The reason for the increase in sensitivity is not known but CSF having a much lower total protein concentration may be a more ideal specimen for the RIA test than is serum.

The patients tested in this study were selected on the basis of methodology testing. Since the control patients were not matched pairs, no statistical comparison of MS and control patients could be made. Nevertheless, when the MS patients having a CSF HAI titer of <2 are compared to the control patients also having a CSF HAI titer of <2 the findings are quite interesting. RIA rubella antibodies were found in the CSF of 11 out of 12 MS patients in contrast to only 2 out of 12 control patients. Simultaneously 6 out of the 12 MS patients, but none of the control patients, had an abnormally low serum/CSF ratio of rubella antibodies. From the observation that a considerable number of MS patients have reduced serum/CSF rubella antibody ratios, it is concluded that there is a local production of rubella virus antibodies in the central nervous system of these patients.

The excellent technical assistance of Mrs. Leena Soini is gratefully acknowledged.

This work was supported by grants from The State Medical Research Council of Finland and the Sigrid Juselius Foundation.

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Acta path. microbiol. scand. Sect. B, 85 117 124 1977

THE CATABOLISM OF GLUCOSE, GLUTAMATE PYRUVATE AND ACETATE IN *NEISSERIA ELONGATA* SUBSP *GLYCOLYTICA*

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Holten, E The catabolism of glucose, glutamate, pyruvate and acetate in *Neisseria elongata* subsp *glycolytica*. Acta path. microbiol. scand. Sect. B, 85 117 124 1977

Activities corresponding to the enzymes glucokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase malate dehydrogenase pyridine nucleotide independent malate dehydrogenase and glutamate dehydrogenase were found in cell free extracts from *Neisseria elongata* subsp *glycolytica*. Activities corresponding to 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase were not found Glucose was catabolized only in the pentose phosphate pathway The radiorespirometric findings suggest an extensive recycling of the triose and fructose phosphates There was no evidence for formation of pyruvate from glucose Glutamate was oxidized in the tricarboxylic acid cycle. Pyruvate and acetate were obviously catabolized by the glyoxylic and tricarboxylic acid cycles, as in *N elongata*.

Key words *Neisseria elongata subsp glycolytica* glucose glutamate pyruvate acetate catabolism.

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Received 17 iii 76 Accepted 12.x.76

The recently isolated rod-shaped *Neisseria elongata* subsp *glycolytica* differs from *N elongata* in colony consistency a positive catalase reaction, and by its ability to make acid from glucose (3 The acid production is small, but significant, and is different from that observed if the other saccharolytic species of *Neisseria* are used

This paper deals with the primary pathways of glucose catabolism, the activity of the tricarboxylic acid cycle and the oxidation of pyruvate and acetate in *N elongata* subsp. glycolytica. The results are compared with those of other species of *Neisseria* (6–12, 14)

MATERIALS AND METHODS

Organisms Blood agar grown cells of *N elongata* subsp glycolytica 6171/75 were used throughout the study

Cell free extracts were made by ultrasonic treatment followed by centrifugation at 10,000 × *g* for 30 minutes, and stored at −20° C (13)

Enzyme assays. *Glucokinase* (ATP D-glucose phosphotransferase E.C. 2.7.1.2) was assayed by coupling this reaction with the endogenous G-6-P dehydrogenase (7) Since the specific activity of G-6-P dehydrogenase appeared to be higher than that of the glucokinase the latter enzyme was considered to be the rate-limiting factor

Abbreviations G-6-P D-glucose 6-phosphate 6-PG 6-phospho-D-gluconate KDPG 6-phospho-2-keto-3-deoxy-D-gluconate

G-6-P dehydrogenase (D-glucose 6-phosphate NADP oxidoreductase E.C 1 1 1 49) and 6-PG dehydrogenase (6-phospho-D-gluconate NADP oxidoreductase E.C. 1 1 1 44) were assayed by following the reduction of NADP as described (7 8)

The assay of 6 PG dehydrase (6-phospho-D-gluconate hydro-lyase, E.C. 4.2 1 12) and KDPG aldolase (6-phospho-2 keto-3-deoxy D-gluconate D-glyceraldehyde-3 phosphate lyase, E.C.4 1.2.14) were measured by analyzing the formed pyruvate by lactate dehydrogenase (8) Since no activity corresponding to KDPG aldolase was found, extract from *N elongata* M2 was included in the reaction mixture in order to be able to detect 6-PG dehydrase activity *N elongata* M2 has KDPG aldolase but not 6-PG dehydrase (8)

Malate dehydrogenase (L-malate NAD oxidoreductase, E.C. 1 1 1.37) and the glutamate dehydrogenases (L-glutamate NAD oxidoreductase (deaminating) E.C. 1 4 1.2 and L-glutamate NADP oxidoreductase (deaminating) E.C 1 4 1 4) were examined by following the reduction or oxidation of coenzymes (13 14)

In the assay for pyridine nucleotide independent malate dehydrogenase the reduction of $K_3Fe(CN)_6$ at 400 nm was followed (12)

Radiorespirometry was done as described (9) Phosphate- NaCl/Tris- and KCl/Tris-based minimal media were used The CO_2 trapping solution was changed every 15 minutes.

Incorporation of label from glucose into pyruvate The reaction mixture contained cell suspension (in NaCl/Tris-based medium) 1 ml specifically labelled glucose (0 2 μCi) 2 μmoles, K_3AsO_3 25 μmoles, and NaCl/Tris-based minimal medium to a volume of 4 ml (16) After 20 minutes of incubation at 37 C the reaction was stopped by 0.4 ml 70 per cent $HClO_4$ Pyruvate was extracted from the solution as the 2 4-dinitrophenylhydrazone and analysed by thin layer chromatography as described (17) Samples to be counted were transferred to counting vials and mixed with 4.5 ml of water and 10 ml of the same scintillation fluid as that used in the radiorespirometry experiments.

Chemicals Specifically ^{14}C-labelled D-glucose, *DL*-glutamate pyruvate and acetate were from the same sources as in previous experiments (9-11) Other chemicals (G-6-P 6-PG KDPG L-malate and pyridine nucleotides) were also those used previously (7 8 14)

RESULTS

1 Enzyme Activity in Cell Free Extracts

Activities of selected enzymes of primary glucose catabolism and tricarboxylic acid cycle were examined (Table 1) The extracts could phosphorylate glucose to G-6-P Activities corresponding to G-6-P dehydrogenase and 6-PG dehydrogenase were also present. No 6-PG dehydrase or KDPG aldolase activity was found

*In other Neisseria species specific differ*ences in the NAD-dependent malate dehydro-

TABLE 1 *Enzyme Activities in N* elongata *subsp* glycolytica

Enzyme	Coenzyme	Activity*
Glucokinase		*10*
Glucose 6-phosphate dehydrogenase		50
6-phosphogluconate dehydrogenase		22
6-phosphogluconate dehydrase		0§
2 keto-3-deoxy-6-phosphogluconate aldolase		0
Malate dehydrogenase	NAD	4
Malate dehydrogenase	NADH	3165
Pyridine nucleotide independent malate dehydrogenase		35
Glutamate dehydrogenase	NAD	36
Glutamate dehydrogenase	*NADH*	20
Glutamate dehydrogenase	NADP	57
Glutamate dehydrogenase	*NADPH*	*150*

* The activity of glucokinase is expressed as nmoles of coenzyme reduced per mg protein during th first minute of incubation. The activities of the pyridine nucleotide-linked enzymes are expressed as nmoles coenzyme reduced or oxidized per minute per mg protein. The activity of the pyridine nucleotide independent *malate dehydrogenase is expressed* as nmoles malate oxidized per minute per mg protein

§ Extract from *N elongata* M2 added to the reaction mixture see Materials and Method

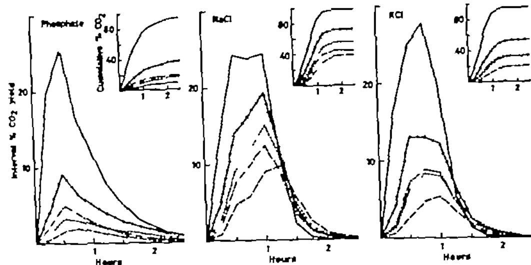


Fig. 1 Radiorespirometric pattern for the utilization of D-glucose by *N. elongata* subsp. *glycolytic* 6171/75 Phosphate: phosphate-based minimal medium. NaCl: NaCl/Tris-based minimal medium. KCl: KCl/Tris-based minimal medium. D-glucose-1-^{14}C ——— D-glucose-2-^{14}C ⊢⊣ D-glucose-3-^{14}C D-glucose-4-^{14}C — — — D-glucose-6-^{14}C — —

genase were found (14). The present strain had the pattern seen in *N. elongata* and the species of the genus *Branhamella* (formerly *N. catarrhalis*, *N. ovis* and *N. caviae*): very low activity in the oxidation of malate and extremely high activity in the reverse reaction. The pyridine nucleotide independent malate dehydrogenase (12) was also present.

Both NAD and NADP could be used as coenzymes in the glutamate dehydrogenase reaction. Just as in *N. elongata* and the other *Neisseria* species two glutamate dehydrogenases, one specific for each coenzyme, were found by electrophoresis of extract from strain 6171/75 (6).

Radiorespirometry

Glucose. Fig. 1 shows the radiorespirometric pattern for the utilization of glucose in different media and Table 2 shows the isotopic recovery at the end of each experiment.

Almost all the radioactivity of C1 appeared as CO_2. The amount of CO_2 formed from the other carbon atoms depended on the medium. In NaCl/Tris-based medium the yield of CO_2 was highest, with very little incorporation of radioactivity in the cells. The highest incorporation of radioactivity was found when phosphate-based medium was used. In this medium the $^{14}CO_2$ yield from the carbon atoms other than C1 was low.

The low yield of CO_2 from C4 indicates that only a small fraction, if any, of the glucose is oxidized via the Embden-Meyerhof or Entner-Doudoroff pathways. The main route of glucose degradation is probably the pentose phosphate pathway. The lack of enzymes of the Entner-Doudoroff pathway is consistent with this. The results suggest recycling of triose phosphates through fructose phosphates to G-6-P.

Glutamate. The radiorespirometric pattern of glutamate oxidation (Fig. 2, Table 2) showed that this amino acid is oxidized via the tricarboxylic acid cycle. The oxidation and incorporation of radioactivity into cells was slightly less in phosphate- than in KCl/Tris-based medium.

Pyruvate and acetate. These compounds were rapidly oxidized and the radiorespirometric patterns (Figs. 3 and 4, Table 3) were consistent with an oxidation via the tricarboxylic acid cycle. However, the extensive con-

TABLE 2 *Utilization of* ^{14}C *Labelled D glucose and DL-glutamate by N elongata subsp* glycolytica *6171/75*

Substrate	Medium	µmoles substrate	Per cent of label incorporated into			Per cent total recovery of label
			CO	Cells	Medium	
D-glucose-1-^{14}C	Phosphate-based minimal medium	0 75	93	4	3	100
D-glucose 2 ^{14}C			37	29	34	98
D-glucose-3 ^{14}C			17	37	46	92
D-glucose-3 4-^{14}C			13	47	40	90
D-glucose-4 ^{14}C*			9	57	34	
D-glucose-6-^{14}C			18	51	31	89
D-glucose-1 ^{14}C	NaCl/Tris-based minimal medium	0.33	98	0	2	106
D-glucose 2 ^{14}C			72	4	24	103
D-glucose 3-^{14}C			57	5	38	108
D-glucose 3 4 ^{14}C			48	8	43	103
D-glucose-4 ^{14}C*			40	11	49	
D-glucose-6-^{14}C			46	10	44	104
D-glucose-1 ^{14}C	KCl/Tris-based minimal medium	1.5	95	3	2	105
D-glucose 2 ^{14}C			54	22	24	104
D-glucose-3-^{14}C			34	29	37	103
D-glucose-3 4 ^{14}C			28	39	33	98
D-glucose-4-^{14}C*			21	48	31	
D-glucose-6-^{14}C			34	38	28	96
DL-glutamate-1 ^{14}C	Phosphate-based minimal medium	15	47	8	45	108
DL-glutamate-2 ^{14}C			28	12	60	100
DL-glutamate-3 4-^{14}C			9	19	72	99
DL-glutamate-5-^{14}C			30	12	58	99
DL-glutamate 1 ^{14}C	KCl/Tris-based minimal medium	10	47	12	41	101
DL-glutamate-2 ^{14}C			34	17	49	99
DL-glutamate-3 4 ^{14}C			12	34	54	99
DL-glutamate-5-^{14}C			35	18	47	101

* Calculated from the data of D-glucose-3 ^{14}C and D-glucose-3 4-^{14}C.
The experiments were run for 2½ hours. After that time the production of CO_2 had ceased.

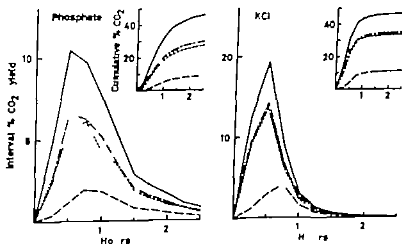
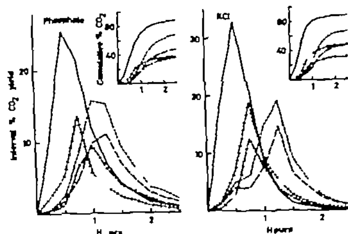


Fig 2 Radiorespirometric pattern for the utilization of DL glutamate by *N elongata* subsp. *glycolytica* 6171/75 in phosphate- and KCl/Tris-based minimal media DL-glutamate-1 ^{14}C —— DL-glutamate-2-^{14}C ······ DL-glutamate-3 4 ^{14}C — — — DL-glutamate-5-^{14}C — —

Fig 3 Radiorespirometric pattern for the utilization of pyruvate by *N. elongata* subsp. *glycolytica* 6171/75 in phosphate- and KCl/Tris-based minimal media in the absence and presence of L-glutamate. Pyruvate-1-^{14}C ——— pyruvate-2-^{14}C .... pyruvate-3-^{14}C — — — pyruvate-2-^{14}C plus glutamate -+-+- pyruvate 3-^{14}C plus glutamate —+—



version of acetate and of the C2 and C3 of pyruvate to CO_2 suggests that also the glyoxylic acid cycle is functioning in this strain. A concurrent operation of both the glyoxylic and the tricarboxylic acid cycles is required to produce CO_2 from acetate when this is the only substrate (21).

The CO_2 formation from pyruvate was not affected by the media used. When glutamate was added, the CO_2 peak appeared earlier but the total yield of $^{14}CO_2$ was reduced. This was also the case when acetate functioned as the substrate.

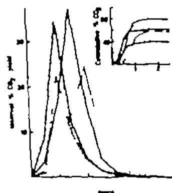


Fig 4 Radiorespirometric pattern for the utilization of acetate by *N. elongata* subsp. *glycolytica* 6171/75 in KCl/Tris-based minimal medium in the absence and presence of L-glutamate. Acetate-1-^{14}C ——— acetate-2-^{14}C .... acetate-1-^{14}C plus glutamate — — — acetate-2-^{14}C plus glutamate —+—

3 *Attempt to Isolate Pyruvate*

The results from the radiorespirometric experiments do not exclude the Embden-Meyerhof or Entner-Doudoroff pathways as possible routes of primary glucose catabolism beside the pentose phosphate pathway. If glucose is catabolized by either of the two former pathways, pyruvate should be an intermediate metabolite. I attempted to isolate pyruvate from cells incubated with specifically labelled glucose in NaCl/Tris-based medium. The cells were poisoned by arsenite to prevent oxidation of pyruvate. Using strain 6171/75 no pyruvate was found after chromatography of the 2,4-dinitrophenylhydrazones, nor was there any radioactivity where the pyruvate spots were supposed to be. In contrast, using cells of *N. meningitidis* pyruvate was readily detected.

DISCUSSION

The saccharolytic *Neisseria* species catabolize glucose via the Entner Doudoroff and pentose phosphate pathways, and oxidize pyruvate via the tricarboxylic acid cycle (9). The non-saccharolytic *Neisseria*, *N. flavescens*, *N. cinerea* and *N. elongata* probably do not catabolize glucose as these species do not form substantial amounts of CO_2 from ^{14}C-labelled glucose (9). *N. elongata* subsp. *glycolytica* however readily makes CO_2 from labelled glucose but its radiorespirometric pattern

TABLE 3 *Utilization of ¹⁴C Labelled Pyruvate and Acetate by N elongata subsp glycolytica 6171/75 in the Absence and Presence of L-glutamate*

Substrate	Medium	µmoles substrate	µmoles glutamate	Per cent of label incorporated into			Per cent total recovery of label
				CO	Cells	Medium	
Pyruvate 1 ¹⁴C	Phosphate-based minimal medium	30	0	87	3	10	100
Pyruvate-2 ¹⁴C				67	18	15	107
Pyruvate-3-¹⁴C				47	29	24	107
Pyruvate-2 ¹⁴C		10	10	37	33	30	97
Pyruvate-3-¹⁴C				35	32	33	105
Pyruvate 1 ¹⁴C	KCl/Tris-based minimal medium	30	0	88	4	8	102
Pyruvate-2 ¹⁴C				67	20	13	101
Pyruvate-3-¹⁴C				47	30	23	99
Pyruvate-2-¹⁴C		10	10	48	35	17	98
Pyruvate-3 ¹⁴C				32	43	25	93
Acetate-1-¹⁴C	KCl/Tris-based minimal medium	10	0	79	12	9	99
Acetate 2 ¹⁴C				57	25	18	96
Acetate-1 ¹⁴C		10	10	61	28	11	98
Acetate-2 ¹⁴C				40	40	20	95

The experiments were run for 2½ hours. After that time the production of CO_2 had ceased.

deviates significantly from that of the other saccharolytic *Neisseria*. Because no pyruvate made from glucose can be found and the CO_2 yield from C4 of glucose is small the participation of the Embden Meyerhof or Entner Doudoroff pathways in glucose catabolism seems negligible. An extensive recycling of pentose phosphate through triose and hexose phosphates might account for the radiorespirometric pattern Fructose phosphate arising in the transaldolase and transketolase reactions will have this labelling pattern

2		2
3		3
2	and	3
4		4
5		5
6		6

where the numbers 2–6 correspond to the numbers of the carbon atoms in the original glucose molecule (18) Triose phosphate formed in the transketolase reactions may also combine in the aldolase reaction to form fructose phosphate with this labelling pattern (18)

6
5
4
4
5
6

Such a recombination of trioses to fructose phosphate probably occurs in the other saccharolytic *Neisseria* (9 19) A recycling of this fructose phosphate through G-6-P to the pentose phosphate pathway might eventually release respiratory CO_2 from the carbon atoms C2–C6 The observed order of $C2>C3\geq C6>C4$ (Table 2) is in fair accordance with such a recycling in the pentose phosphate pathway In other saccharolytic *Neisseria* the conversion of triose to pyruvate was inhibited in some of the media used (9) This seems to be the case in all media examined with the present strain but whether the reactions are completely blocked or the amount of pyruvate made is too small to be detected is still an open question However the results suggest that the primary glucose catabolism in strain 6171/75 does not occur via the Embden Meyerhof or Entner Doudoroff pathways.

I have calculated the maximal yield of CO from C4 (17) and compared this with the observed yield (Table 4). *Kitos et al.* (17) based their calculation on observations of *Acetobacter suboxydans* which apparently catabolizes almost all the offered glucose by a recycling in the pentose phosphate pathway. The small discrepancies between the observed and the calculated values—highest in NaCl/Tris-based medium where the catabolic activity is greatest—may be due to the function of pathways other than the pentose phosphate pathway.

TABLE 4. *Calculated and Observed Yield of CO from C4 of Glucose*

Medium	Per cent yield of CO from C4	
	Calculated	Observed
Phosphate-based minimal medium	7	9
NaCl/Tris-based minimal medium	34	40
KCl/Tris-based minimal medium	19	21

The calculated percentages are maximal values, and are calculated from the CO yield from C6, using the formula of *Kitos et al.* (17).

The fraction of radioactivity from glucose incorporated into cellular components is greatest in phosphate-based medium, less in KCl/Tris-based medium. Accumulation of phosphorylated compounds might account for this difference, however I have not attempted to isolate metabolic products other than pyruvate.

It is apparently not clear which product of glucose metabolism causes the acid reaction. In *N. meningitidis* (15) and *N. gonorrhoeae* (1) acetate accumulates. Probably neither lactate nor acetate account for the acid reaction produced by strain 6171/75 because no pyruvate was found, although the formation of acetate by metabolic pathways not including pyruvate cannot be completely excluded.

Data obtained by radiorespirometry of glutamate show that the tricarboxylic acid cycle is an important pathway also in the strain examined. The pattern is similar to that found in the other *Neisseria* species, as well as in the species of *Branhamella* (10).

The radiorespirometric pattern of pyruvate and acetate oxidation, via the glyoxylic and tricarboxylic acid cycles, resembles that of *N. elongata* and the *Branhamella* species (11). Glutamate lowers the CO yield from acetate and C2 and C3 of pyruvate, which has been explained as the result of a dilution of radioactive intermediates by inactive compounds derived from glutamate (11).

In particular the activity of malate dehydrogenase is similar to that observed in *N. elongata* (14), but also the other enzyme activities resemble those found in *N. elongata* (6–8, 12). The close relationship of strain 6171/75 to *N. elongata* has also been confirmed by genetic methods (3, 5) and by gas chromatography (5). Immunological and electrophoretic studies of the glutamate dehydrogenases further confirm the close affinity to *N. elongata* (3).

N. elongata subsp. *glycolytica* lacks KDPG aldolase which is present in *N. elongata* (8). The only difference in the enzymic activities tested is then found in the Entner Doudoroff pathway which is already blocked because of lack of 6-PG dehydrase. The present findings cannot tell us why strain 6171/75 makes CO_2 from glucose while *N. elongata* does not. May be the explanation is a different permeability to glucose, but this point has not been tested.

Less attention should be paid to the carbohydrate fermentation pattern as a criterion in the classification of *Neisseria* (2, 20). The consequence of this has been taken into consideration in the case of the species *N. flava*, *N. subflava* and *N. perflava* which have been reclassified as subspecies of *N. subflava* (4, 20). The genetic and chemical data were accordingly considered more important than the fermentation of glucose when the taxonomic status of strain 6171/75 was discussed, and the strain was classified as a subspecies of *N. elongata* (5). The present results also indicate that strain 6171/75 is more closely related to *N. elongata* than to any other species of *Neisseria*.

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Acta path. microbiol. scand. Sect. B, 85 125–128, 1977

DEMONSTRATION OF HOST ANTIGENS IN THE MYXOVIRUS MEMBRANE LYSIS OF VIRUS BY ANTIBODY AND COMPLEMENT

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Haukenes, G Demonstration of host antigens in the myxovirus membrane: Lysis of virus by antibody and complement. Acta path. microbiol. scand. Sect. B, 85 125–128, 1977

Complement-mediated lesions developed in myxovirus membranes in the presence of antibody to host antigens. Intact virions reacted with antibody to the keratosulphate-like host antigen present in the spike layer. Spike-deprived influenza virus reacted in the same way when an antiserum to the Forssman glycolipid was used. The holes in the membrane varied considerably in size, but were otherwise similar to those found in erythrocyte membranes. This immune electron microscopy method proved useful for the localization of host material in viral membranes.

Key words: Myxovirus; host antigen; electron microscopy; complement lesions.

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Received 21.x.76 Accepted 3.xi.76

Evidence has earlier been presented that influenza virus grown in the allantoic cavity of embryonated hens' eggs acquires two host antigens: the keratosulphate-like allantoic host antigen (3, 4) (here termed host antigen, h.a.) in the spike layer and the Forssman glycolipid in the lipid layer of the membrane (6).

In the present study we have examined intact and spike-deprived myxovirions for complement-mediated membrane lesions.

MATERIALS AND METHODS

Virus

Influenza A virus PR/8/34 (H$_0$N$_1$), influenza B virus strain Lee and para-influenza 1 virus (Sendai) were used. The viruses were propagated in the chick allantoic cavity for 2–3 days. To avoid interference from free h.a. in the allantoic fluid, the virus was purified either by adsorption to and elution from guinea pig erythrocytes (virus eluate) or by pelleting in the ultracentrifuge at 100,000 × g for 45 min.

Removal of spikes from influenza virus A/PR/8 was accomplished by treatment with the protease bromelain (2). The enzyme (E.C. 3.4.4.24 Sigma) was mixed with the virus to a final concentration of 1 mg/ml with 0.01 M dithiothreitol (Sigma) added. The action of the enzyme was stopped by pelleting the virus particles at 0° C at 100,000 × g for 45 min. Control virus was treated with the reducing agent only.

Host Antigen (h.a.)

The material was purified from normal allantoic fluid as described earlier (4). One mg of purified material blocked 4 haemagglutination inhibition (HI) doses in the B/Lee anti A/PR/8 system to dilution of 1 in 32,000 (cf. below).

Antisera

Antiserum against influenza A virus PR/8 was raised in rabbits by means of intravenous injections of a virus eluate. This antiserum gave an HI titre



Fig 1 a. Multiple lesions in Sendai virus membranes after treatment with antibody to h.a. and complement. Bar- 100 nm



Fig 1 c "Collapsed" Sendai virus particle (treated as 1 a) with multiple holes of varying sizes. Bar 100 nm.



Fig 1 b Radially arranged ub-units of the border lining the complement mediated hole in a Sendai virus membrane H gher magnification of 1 a. Bar 100 nm.



Fig 1 d Spontaneous lesions in a Sendai virus membrane (control without antibody and complement) Bar 100 nm.

of 512 against a virus eluate of Influenza B/Lee virus. This heterologou HI reaction was solely caused by the shared h a since purif ed h a completely blocked the HI if added in advance to the serum. The rabb t anti h.a antiserum was obta ned by subcutaneous injections of purif ed h.a in Freunds complete adjuvant.

Antiserum to the Forssman antigen was produced as described earlier (6)

Immune Electron Microscopy

The virus was concentrated by ultracentrifugation to gi e more than 20 virus particles per square of the grid in the final mixture The anti PR/8 antiserum and the anti-h.a. antiserum were pre-treated with 5 volumes of RDE (neuraminidase from V cholerae filtrate, Flow Labs.) over night at 37 C followed by incubation at 56 C for 1 h Equal amounts of virus and antiserum (diluted 1 10) were mixed and incubated at 4 C overnight Complement ("active guinea pig serum) was then added to a final concentration of 1 15 and the mixture was further incubated at 37 C for 1 h The anti-Forssman antiserum was heat inactivated (56 C, 30 min) and used in dilution 1.50 without any further pre treatment.



Fig. 2 Spike-less influenza A particle treated with anti-Forssman antiserum and complement. Bar 100 nm.

The preparations were stained with 1 per cent potassium phosphotungstate, pH 7.0, on the grids (no 400) and examined with Hitachi HU 12 A electron microscope. Controls were virus with complement only with antiserum only and without both.

RESULTS

Intact Virus

When Sendai virus was treated with anti-A/PR/8 antiserum and complement, the most conspicuous feature was the high proportion of damaged virions as compared with that in controls without antibody or complement. The damaged particles were swollen and had taken up the negative stain. Very fine holes or craters, ranging in diameter from 10 to 60 nm, were seen in the membranes of many of the damaged particles (Fig 1 a, b and c). Similar holes were never found in the absence of complement. Some particles seemed to be empty and "collapsed" and were obviously devoid of nucleoprotein (Fig 1 c).

The complement-mediated holes were easily distinguishable from spontaneous membrane lesions which develop in the absence of complement and antibody. These lesions were of irregular shape and no limiting border could be seen (Fig 1 d). In contrast, the complement-mediated holes were circular or oval with a "raised" and sharply outlined limiting border. At high magnification (Fig 1 b) the border seemed to consist of radially arranged sub-units. Identical results were obtained if influenza virus and the antiserum to purified h.a. were used.

Intact influenza A virus did not react with anti-Forssman antiserum and complement. When the virus was deprived of spikes, typical holes in the membrane appeared, as described above (Fig 2).

The reactivity of the anti-h.a. and anti-Forssman antisera could be blocked by pre-treatment of the antisera with purified h.a. and ground guinea pig kidney respectively.

DISCUSSION

Complement-mediated holes in the membrane in the presence of antibody have been described in an earlier study where avian infectious bronchitis virus was used (1). In the present study this method was found to be well-suited for the demonstration and localization of host material in viral membranes of two myxoviruses.

The raised border of the complement-mediated holes had the same fine structure as the lesions observed in erythrocyte membranes (5). In erythrocytes, this border is considered to be built-up of the haemolytic complex (C 5–9) of the complement proteins (7).

Studies using this method are in progress to see whether other egg-grown viruses also acquire the h.a.

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YERSINIA ENTEROCOLITICA AND *YERSINIA* LIKE MICROBES ISOLATED FROM MAMMALS AND WATER IN NORWAY AND DENMARK

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Kapperud, G. *Yersinia enterocolitica* and *Yersinia* like microbes isolated from mammals and water in Norway and Denmark. Acta path. microbiol. scand. Sect. B, 85: 129–135, 1977.

Data pertaining to 149 strains belonging to genus *Yersinia* are summarized in this paper. *Yersinia enterocolitica* (Y.e.) was isolated from the faeces of 31 of 305 small rodents and from 5 of 31 shrews (Soricidae) trapped at five localities in Norway and one locality in Denmark. Isolations were obtained from 9 of 29 water samples collected within the trapping areas. Three of 25 red foxes (*Vulpes vulpes*) from one locality in Norway harbored Y.e. in their faeces. Y.e. serotype 16 was isolated from one zoologist suffering from diarrhoea. A total of 85 strains from small rodents, shrews, water and foxes showed biochemical properties intermediate to Y.e. and *Y. pseudotuberculosis*. Another three strains were classified as *Y. pseudotuberculosis* on biochemical basis. They were obtained from small rodents in Denmark. Serological examination of 59 small rodents naturally infected with Y.e. and related microbes, revealed two cases of low antibody titres (80) against homologous isolates. Pathological examination of 44 of these animals gave a negative result. Strains antigenically related to the same serotypes were frequently isolated from both terrestrial ecosystems and adjacent freshwater. Strains related to serotype 6 dominated both in red fox and in their small rodent prey. No evidence was found for a dynamical significance of Y.e. in populations of small rodents. The results indicate that Y.e. and *Yersinia* like microbes have a broad occurrence in terrestrial ecosystems.

Key words: *Yersinia enterocolitica*; *Yersinia* like microbes; mammals; water.

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Received 5.xi.76. Accepted 5.xi.76.

The present article represents the second part of a research program with the aim of assessing small mammals as reservoirs for *Yersinia enterocolitica* (Y.e.) and with the purpose of studying Y.e. as a potential factor in the population dynamics of small rodents. The occurrence of Y.e. in the faeces of small rodents from widespread localities in Fennoscandia has previously been documented (5). This study included the isolation of two strains antigenically related to O-serotype 3. Both differed biochemically, however, from strains currently associated with disease in humans.

The results obtained in the first part of this research program did not allow any definite conclusions concerning the clinical signi-

ficance of Y e. in the small rodent hosts. This problem is subjected to closer examination in the present study It was speculated that latent infections with Y e. may become manifest under conditions of stress such as occur during the population peak and the subsequent decline of small rodents in the northern ecosystems. During 1974 the small rodent populations in southern Norway reached peak densities and declined through the winter and early spring of 1975 Thus, an opportunity was provided to examine animals from various stages of the small rodent cycle

The dense populations of small rodents during peak years may facilitate the spread of Y e. Predators such as cats and foxes may become infected by ingestion of infected prey Faecal contamination of food and drinking water might also contribute to the propagation of this kind of yersiniosis. In the present study an attempt is made to trace individual serotypes through a food chain from small rodent prey to fox predators. Likewise, a comparison is performed between strains obtained from terrestrial ecosystems and from adjacent freshwater

MATERIALS AND METHODS

The material is presented in Table 1

I Collections

Nine species of small rodents and shrews (Soricidae) were live trapped at five localities in southern Norway and one locality in Denmark. Table 1 lists the localities. The species are shown in Table 2 Three of the trapping areas (A, B C) have been described previously (5) In addition, samples were taken from

D Mols Ecological Research Station, Denmark Trapping stations Woods dominated by beech bordering pastures

E. Mosstrond in Telemark, Norway Trapping stations Subalpine birch forest surrounding small farms.

The traps were heat sterilized prior to each catch Faeces and plasma samples were obtained as specified elsewhere (5)

A total of 29 water specimens were collected from brooks and ponds within the trapping areas. Twenty five red foxes (*Vulpes vulpes*) were shot at Hedal in Oppland, Norway during the winters 1973/74 and 1974/75 Faeces were obtained by rectal swabs. Faeces samples were also taken from six zoologists working with small rodents at the University of Oslo, Norway

II Processing Collected Material

Faecal specimens were transported and cultivated individually as specified earlier (5) Water samples were cultured on lactose broth for ordinary bacteriological examination within 48 hours. Broths were incubated at 37 C for 46–48 hours. When growth occurred (with or without gas formation) secondary inoculations were made on LSU agar The plates were incubated at room temperature for 46–48 hours.

Pure culture was made of all colonies showing morphological similarity to Y e Urease positive cultures were selected, and a primary biochemical characterization was carried out by the three tube method described by *Lassen* (8) The subsequent procedure including further biochemical characterization and serotyping, has been described elsewhere (5)

In the present study the following six biochemical differentiae were regarded diagnostic of *Y pseudotuberculosis* (Y ps.) as compared to *Y enterocolitica* (Y e) Fermentation of rhamnose and melibiose no fermentation of sucrose, cellobiose and sorbose and absence of ornithine decarboxylase. Strains which otherwise possessed biochemical properties consistent with those of genus *Yersinia* were identified as follows

Exhibiting all of the six differentiae – – Y ps
Exhibiting none of the six differentiae – – Y.e
Exhibiting 1–5 of the six differentiae – – *Yersinia* like microbes.

Cultures of Y ps. and *Yersinia* like microbes were agglutinated against Y ps. O-antisera I-V in addition to the O-antisera of Y.e. A titre of 160 or higher was considered indicative of antigenical relationship of the isolated strain to that particular serotype.

III Reaction of the Host

Plasma from 59 small rodents naturally infected with Y e or *Yersinia* like microbes were tested by tube agglutination against homologous isolates Thirty eight of these animals were investigated for macroscopic pathological changes. The animals had been stored at –20 C for up to two years before autopsy was performed. Liver and spleen from another six small rodents naturally infected with Y.e. were sectioned, stained with hematoxylin/eosin and submitted to histopathological examination.

RESULTS

The results are presented in Table 1–4 and in the text.

I Y. enterocolitica (Y.e.)

A total of 61 strains were classified as Y.e. on a biochemical basis. Fifty two of these strains were antigenically related to one or more Y.e. O-serotype (Table 2). Only 19 strains could be ascribed to one of the biotypes proposed by *Wauters* (12). 17 strains belonged to biotype 1. The other strains showed various deviations from the biotype scheme. Deviations occurred most frequently in the reactions with xylose and lactose.

Strains antigenically related to serotype 6 constituted 44 per cent of the total number of Y.e. cultures isolated (Table 2). Three of the four strains obtained from red fox belonged to this serotype which also dominated among their small mammal prey. Four strains were antigenically related to serotype 3. They were obtained from three different species of small rodents captured at the same locality (Kviteseid, Norway). Biochemically these strains resembled biotype 1 but deviated in the reactions with xylose and lactose. Y.e. was isolated from eight of the nine small mammal species and from all five localities examined. Simultaneous isolations of Y.e. from small mammals and adjacent freshwater were obtained in locality A and C (Table 1). The frequency of small rodents harboring Y.e. was not significantly correlated to the population density of these animals.

A high diversity of serologically and biochemically different strains were found in Kviteseid, the locality where the majority of field work was concentrated. In Table 3 the serological properties of strains isolated from small mammals in this locality are compared with strains obtained from brooks running through the trapping area.

II Y. pseudotuberculosis (Y. ps.)

Three strains were classified as Y. ps. on biochemical basis. They were obtained from small rodents (*Clethrionomys glareolus*) in Mols, Denmark. Antigenically these strains were related to Y. ps. O-serotype IIB and to Y.e. O-serotype 4 and 21.

TABLE 1. Y. enterocolitica in *Faeces of Mammals and Water from Norway and Denmark. Presentation of the Material. Number of Samples Harboring* Y. enterocolitica

Locality*	Date	No. of samples				
		Small rodents	Shrews	Water	Red fox	Humans
A. Kviteseid	Oct. 1974	63 (7)	–	4 (1)	–	–
	Nov. 1974	46 (7)	4 (0)	4 (1)	–	–
	Jan. 1975	9 (1)	18 (3)	4 (0)	–	–
	May 1975	8 (4)	2 (1)	4 (2)	–	–
	Oct. 1975	19 (2)	1 (1)	4 (3)	–	
B. Vassfaret	Sept. 1974	38 (1)	5 (0)	4 (0)	–	–
C. Fuse	Oct. 1974	60 (3)		2 (1)	–	–
D. Mols	Dec. 1974	49 (6)	1 (0)	2 (0)	–	–
E. Mosstrand	Jan. 1975	13 (0)	–	1 (1)	–	–
F. Hedal	1973–1975	–		–	25 (3)	–
G. Oslo	Oct. 1974		–	–	–	6 (1)
Sum		305 (31)	31 (5)	29 (9)	25 (3)	6 (1)

The figures in parentheses present the number of samples harboring *Y. enterocolitica*.
* A, B, C, E, F, G – Norway. D – Denmark.

ficance of Y e. in the small rodent hosts. This problem is subjected to closer examination in the present study It was speculated that latent infections with Y e. may become manifest under conditions of stress such as occur during the population peak and the subsequent decline of small rodents in the northern ecosystems. During 1974 the small rodent populations in southern Norway reached peak densities and declined through the winter and early spring of 1975 Thus, an opportunity was provided to examine animals from various stages of the small rodent cycle

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Pure culture was made of all colonies showing morphological similarity to Y e. Urease positive cultures were selected, and a primary biochemical characterization was carried out by the three tube method described by *Larsen* (8) The subsequent procedure including further biochemical characterization and serotyping has been described elsewhere (5)

In the present study the following six biochemical differentiae were regarded diagnostic of *Y pseudotuberculosis* (Y ps.) as compared to *Y enterocolitica* (Y e.) Fermentation of rhamnose and melibiose no fermentation of sucrose cellobiose and sorbose and absence of ornithine decarboxylase. Strains which otherwise possessed biochemical properties consistent with those of genus *Yersinia* were identified as follows

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III Reaction of the Host

Plasma from 59 small rodents naturally infected with Y.e or *Yersinia* like microbes were tested by tube agglutination against homologous isolates. Thirty eight of these animals were investigated for macroscopic pathological changes. The animals had been stored at −20 C for up to two years before autopsy was performed. Liver and spleen from another six small rodents naturally infected with Y.e. were sectioned stained with hematoxylin/eosin and submitted to histopathological examination.

TABLE 4 *Yersinia Like Microbes in Small Mammals and Water Samples from Norway and Denmark. Biochemical Characterisation*

No. of strains		Biochemical parametres					
Total	Antigenically related to Y.e. O-serotypes	Rhamnose	Melibiose	Sucrose	Cellobiose	Sorbose	ODC
45	31	—	—	(—)	+	+	+
9	5	(+)	—	+	+	+	+
5	2	(+)	—	(—)	(—)	(—)	+
4	3	(+)	(+)	+	+	+	+
3	3	—	—	(—)	+	+	(—)
3	0	(+)	(+)	(—)	(—)	(—)	+
2	1	—	(+)	(—)	+	+	+
2	1	—	—	+	+	+	(—)
2	0	(+)	—	(—)	(—)	+	+
2	0	—	—	+	+	(—)	(—)
1	1	(+)		+	+	(—)	+
1	1	—	(+)	+	+	+	+
1	0	—	(+)	+	+	(—)	+
1	0	—	(+)	(—)	+	+	(—)
1	0	(+)	(+)	(—)	(—)	+	+
1	0	(+)	—	(—)	(—)	(—)	(—)
1	0	—	—	+	(—)	(—)	(—)
1	0	—	—	+	(—)	(—)	+
Sum 85	48						

The parantheses show the characters which indicate biochemical relationship to *Y. pseudotuberculosis.* The characters shown without parentheses is consistent with the properties of *Y. enterocolitica.*
Fermentation of rhamnose, mellibiose, sucrose, cellobiose and sorbose. Presence of ornithine decarboxylase (ODC)

III Yersinia Like Microbes

A total of 85 strains deviated biochemically from the typical Y.e. scheme, showing relationship to Y.ps. They displayed a broad spectrum of biochemical properties intermediate to Y.e. and Y.ps. (Table 4). Fourty eight of these strains were antigenically related to one or more Y.e. O-serotype. No relationship to the O-serotypes of Y.ps. were detected.

II Concurrent Infections

Concurrent infections with two serologically distinct strains of Y.e. or *Yersinia* like organisms were found in 13 small rodents, 1 shrews, two foxes and two water samples. One bank vole (*Clethrionomys glareolus*) from Mols, Denmark, harbored five serologically different strains, one of which could be classified as Y.e.

I Reaction of the Host

Serological examination of 59 small rodents naturally infected with Y.e. or *Yersinia* like microbes revealed two cases of low antibody titres against homologous isolates. Two animals (*Microtus agrestis*) from Kviteseid showed a titre of 80. Both harbored strains antigenically related to Y.e. serotype 1. The strains were sucrose negative. No macroscopical pathological changes were found in these rodents. An additional seven cases of titre 40 and 20 were not considered significant for the demonstration of specific antibodies.

Autopsy of 38 naturally infected small rodents revealed no macroscopically visible pathological changes.

Histopathological examination of spleen and liver from another six small rodents gave a negative result.

TABLE 2. Y enterocolitica *Isolated from Norway and Denmark Serological Characterisation and Origin of Strains*

Relationship to O-serotype	No. of strains	Origin*
1	1	Cg
3	4	Cg Ma, At
4	5	Cg Sa, W
5	1	W
6	26	Cg Ma, As, Af Sa, Sm, Vv W
7	4	Cg Sa
12	2	Sa, Sm
14	1	W
15	1	W
16	3	Cg, H
17	1	W
4 16 18 21§	3	Ll W
–†	9	Ll, Cg As, Vv
Sum	61	

* Key to the abbreviations
Small rodents Cg = *Clethrionomys glareolus* As = *Apodemus sylvaticus* Af = *Apodemus flavicollis* Ma = *Microtus agrestis* Ll = *Lemmus lemmus* At = *Arvicola terrestris* (*Microtus oeconomus*— no isolations obtained)
Shrews Sa = *Sorex araneus* Sm = *Sorex minutus*
Other W = Water H = Human Vv = *Vulpes vulpes* red fox.
§ Agglutinated by more than one of the antisera 4 16 18 and 21
† No relationship to serotype 1–34

TABLE 3 Y enterocolitica *and* Yersinia *Like Microbes Isolated from Kvitsøy Norway Comparison of Strains from Small Mammals and Water*

Relationship to O-serotype	No. of strains isolated from*		
	Small rodents	Shrews	Water
1	1 (2)	–	–
3	4	–	–
4	3	1	1 (1)
5	–	–	1
6	11	2	5
7	1	2	–
11	–	(3)	(1)
12	1 (2)	2 (2)	(3)
15	–	–	1
16	1 (2)	(1)	–
17	–	(1)	1 (1)
28	–	(4)	(2)
4 16 18 21 22§	–	–	1 (3)
–†	4 (5)	(9)	(9)
Sum	26 (11)	7 (20)	10 (20)

* In parentheses The number of strains classified as *Yersinia* like microbes on basis of biochemical properties (see the text) Without parentheses *Y enterocolitica*.
§ Agglutinated by more than one of the antisera 4 16 18 21 and 22
† No relationship to serotype 1–34

TABLE 4 *Yersinia Like Microbes in Small Mammals and Water Samples from Norway and Denmark. Biochemical Characterization*

No. of strains		Biochemical parametres					
Total	Antigenically related to Y.e. O-serotypes	Rhamnose	Melibiose	Sucrose	Cellobiose	Sorbose	ODC
45	31	—	—	(—)	+	+	+
9	5	(+)	—	+	+	+	+
5	2	(+)	—	(—)	(—)	(—)	+
4	3	(+)	(+)	+	+	+	+
3	3	—	—	(—)	+	+	(—)
3	0	(+)	(+)	(—)	(—)	(—)	+
2	1	—	(+)	(—)	+	+	+
2	1	—	—	+	+	+	(—)
2	0	(+)	—	(—)	(—)	+	+
2	0	—	—	+	+	(—)	(—)
1	1	(+)	—	+	+	(—)	+
1	1	—	(+)	+	+	+	+
1	0	—	(+)	+	+	(—)	+
1	0	—	(+)	(—)	+	+	(—)
1	0	(+)	(+)	(—)	(—)	+	+
1	0	(+)	—	(—)	(—)	(—)	(—)
1	0	—	—	+	(—)	(—)	(—)
1	0	—	—	+	(—)	(—)	+
Sum 83	48						

The parantheses show the characters which indicate biochemical relationship to *Y pseudotuberculosis.* The characters shown without parentheses is consistent with the properties of *Y enterocolitica.*
Fermentation of rhamnose, melibiose sucrose, cellobiose, and sorbose. Presence of ornithine decarboxylase (ODC)

III Yersinia Like Microbes

A total of 83 strains deviated biochemically from the typical Y.e. scheme, showing relationship to Y.ps. They displayed a broad spectrum of biochemical properties intermediate to Y.e. and Y.ps. (Table 4). Fourty eight of these strains were antigenically related to one or more Y.e. O-serotype. No relationship to the O-serotypes of Y.ps. were detected.

IV Concurrent Infections

Concurrent infections with two serologically distinct strains of Y.e. or *Yersinia* like organisms were found in 13 small rodents, 12 shrews, two foxes and two water samples. One bank vole (*Clethrionomys glareolus*) from Mola, Denmark, harbored five serologically different strains, one of which could be classified as Y.e.

V Reaction of the Host

Serological examination of 59 small rodents naturally infected with Y.e. or *Yersinia* like microbes revealed two cases of low antibody titres against homologous isolates. Two animals (*Microtus agrestis*) from Kvitesæd showed a titre of 80. Both harbored strains antigenically related to Y.e. serotype 1. The strains were sucrose negative. No macroscopical pathological changes were found in these rodents. An additional seven cases of titre 40 and 20 were not considered significant for the demonstration of specific antibodies.

Autopsy of 38 naturally infected small rodents revealed no macroscopically visible pathological changes.

Histopathological examination of spleen and liver from another six small rodents gave a negative result.

Y. e. antigenically related to serotype 16 was isolated from the faeces of one zoologist working with small rodents. The patient suffered from diarrhoea. No antibody response against this strain was detected. The symptoms appeared after a period of field work at Finse (area C) where Y. e. had previously been isolated from lemmings (*Lemmus lemmus*) and drinking water. Bacteriological examination of faeces from the same person one week before the visit to Finse revealed a normal intestinal flora.

DISCUSSION

Hacking & Sileo (4) suggested that the non-avian wildlife reservoir for Y. e. is far greater than currently recognized. The present results strongly support this opinion. Y. e. and related microbes seem to have a broad occurrence in terrestrial ecosystems. Strains antigenically related to the same serotypes were frequently isolated from both terrestrial ecosystems and adjacent freshwater. The direction of transmission, however, whether from water to terrestrial animals, the reverse, or both, has not been established. Strains antigenically related to serotype 6 dominated both in red fox and in their small mammal prey, indicating transmission along the food chain.

Lassen (7) expressed the opinion that the individual serotypes of Y. e. have their own characteristic reservoirs, each comprising only a very small number of animal species. Recent reports (1, 4, 5, 10) seem to refute this, indicating more extensive reservoirs. The present results point in the same direction. However, the available data are consistent with the view that the clinical significance (if any) of each serotype is confined to a very limited number of host species.

The serological and pathological results do not suggest any clinical importance of the isolated strains for small rodents. An exception can probably be made for certain strains antigenically related to serotype 1. Furthermore, no indicative relationship was detected between the density of small rodents and the prevalence of infection in these animals. Thus no evidence was found for a dynamical significance of Y. e. in populations of small rodents.

The occurrence of biochemically atypical strains of Y. e. has frequently been documented in litterature (2, 3, 9, 11, 13). *Winblad* (14) advocated that there are *Yersinia*-like organisms which should be differentiated from Y. e. In Norway, atypical strains have previously been reported from drinking water (7) and brown trout (6). The present report includes strains constituting a broad spectrum of biochemical properties intermediate to Y. e. and Y. ps., thus presenting the problem of defining the boundaries between these species. Such strict species definitions are artificial as viewed from the concept of a dynamically evolving microbial species. For practical purpose, however, rigid definitions were chosen in agreement with the type concept of classical taxonomy. The less "typical" strains were provisionally classified as *Yersinia*-like microbes. Before any decision can be made concerning the taxonomical allocation of these isolates, the following questions should be answered: Does a continual series of intergrading forms exist between Y. e. and Y. ps., or are there discontinuities in the spectrum? Can one or more separate taxonomical units be recognized between Y. e. and Y. ps.? These questions will be subject to a separate examination comprising a larger material.

Based on this and previously published investigations (5) the following data have been obtained: Natural infections with Y. e. was detected in 43 (9 per cent) of 459 small rodents from widespread localities in Fennoscandia. Y. e. has been isolated from all nine localities and from eight of the nine small rodent species examined. Only few strains showed relationship to serotypes which are known to have clinical significance. For example, six animals (one per cent) harbored strains antigenically related to serotype 3, all originated from the same locality (Kviteseid). Four animals yielded strains related to serotype 1. All these cultures differed biochemically, however, from strains isolated in con

nection with disease in man and domestic animals. Therefore, in view of our present knowledge on the pathogenicity of Y.e., it does not seem likely that small rodents play any decisive role in the epidemiology and epizootology of this kind of yersiniosis.

As data accumulate however the clinical importance of serotypes and biotypes other than those currently associated with disease may become apparent. For instance, *Toma* (10) isolated serotype 6–30 from four children with gastroenteritis. Likewise, the present results include the recovery of Y.e. serotype 16 from a zoologist suffering from diarrhoea. The data presented by *Hacking & Sileo* (4) suggest that serotype 5–27 6–30 and 4–33 may be pathogenic in some species of wildlife.

On the other hand, isolation of Y.e. from diseased individuals does not necessarily reflect a causal connection. Considering the broad representation of Y.e. and related microbes in nature, it is probable that some types are normal elements in the intestinal flora of certain species including man.

I want to thank *Jørgen Lassen* M.D. and chief technologist *Jorunn S ... ader* for their advice. I am also indebted to *Gunnar Holt* D.V.M., and *Rikard Tollman* D.V.M. for performing the pathological examinations.

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Acta path. microbiol scand. Sect. B 85 136–142 1977

CLASSIFICATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI IN THE DIAGNOSTIC LABORATORY

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Oeding P & Digranes A. Classification of coagulase-negative staphylococci in the diagnostic laboratory Acta path. microbiol scand Sect. B 85 136–142 1977

One hundred and ninety-eight coagulase-negative staphylococci isolated from urines, blood cultures, and pus samples were classified by means of two identification schemes, and their wall teichoic acids were determined serologically *S epidermidis S saprophyticus* and *S cohnii* were identified reliably by the use of five criteria acid aerobically from sucrose, trehalose and mannitol phosphatase production, and sensitivity to novobiocin. Further species, notably *S haemolyticus* and *S hominis* could be identified when haemolysis on blood agar plates was included in the criteria group The investigation shows, that a considerable number of coagulase-negative staphylococci isolated from human specimens belong to species other than *S epidermidis* and *S saprophyticus* These staphylococci can cause human infections and should be identified in the diagnostic laboratory *S epidermidis* and *S saprophyticus* were found to contain the teichoic acids previously identified in these species. *S cohnii* contained the same teichoic acids as *S saprophyticus* No characteristic teichoic acid was demonstrated in the other species, but several strains contained poly C (β-N acetylglucosaminylglycerol teichoic acid)

Key words Staphylococci coagulase negative classification.

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Received 1.xii 76 Accepted 1 xn 76

Staphylococcus saprophyticus often causes urinary tract infections seen in young female out patients and should be identified in the bacteriological laboratory Post-cardiac surgery endocarditis and septicaemia caused by coagulase negative staphylococci have increased in significance. Several new staphylococcal species have recently been described taxonomically (4 11) As the differentiation of *Micrococcaceae* is probably rather primitive in most diagnostic laboratories, recent developments make a re-evaluation of our diagnostic efforts desirable.

We have previously reported a simplified scheme for the identification of coagulase-negative staphylococci isolated from urinary tract infections (2) Subsequently five criteria were recommended by the *Subcommittee* (13) for the identification of the clinically most important coagulase-negative species. The purpose of the present investigation was to compare the two identification schemes using collections of coagulase-negative staphylococci isolated from different types of infections, and to evaluate whether species other than *S epidermidis sensu stricto* (11) and *S saprophyticus* are of clinical

interest and therefore should be identified in the diagnostic laboratory

MATERIALS AND METHODS

Strains

Three collections of coagulase-negative staphylococci, totally 198 strains, were examined

1 Coagulase-positive staphylococci, micrococci and some other strains were excluded from the previously reported collection of urinary *Micrococcaceae* (2) isolated in the diagnostic laboratory leaving 128 independent coagulase-negative strains for the present investigation. One hundred and fifteen strains were present in significant numbers, 23 in mixed culture whereas 13 strains were found in insignificant numbers. The strains were from both in- and out-patients (see 2)

2. Forty-seven independent strains were isolated in the diagnostic laboratory from 42 specimens of blood culture and pus.

3 Twenty strains received several years ago from *Dr Harriet Smith* University of Toronto and three strains from *D I M Smith* University of Iowa, had been isolated from blood cultures and pus (three strains) The majority of the patients with positive blood culture had post-cardiac surgery endocarditis.

All strains except those belonging to *S saprophyticus* produced acid from glucose anaerobically

Biochemical Test

Coagulation of rabbit plasma in tubes, and glucose fermentation, were examined according to the procedures recommended by the *Subcommittee* (12) Acid production aerobically from mannitol, trehalose and sucrose was tested on solid media. Arginine dihydrolase activity was examined according to the technique described by *Møller (6)* *Baird-Parker's* phosphatase test on solid medium (3) has been criticized for not being sensitive enough (1) Therefore all the strains were also tested with *Pennock & Huddy* method in fluid medium (9) modified by *Schleifer & Kloos* (11) and with method (A) developed by *Satta et al* (10) and the results of the three methods were compared

Novobiocin Sensitivity

Novobiocin Neo-Sensitabs (Rosco) were used on PDM Antibiotic Sensitivity Medium (AB Biodisk) with horse blood. Inhibition zones ≤27 mm, corresponding to MIC >1.5 µg/ml, were recorded as resistance

Teichoic Acids

The type of wall teichoic acid was determined by double diffusion in agar using reference systems consisting of rabbit immune sera and polysaccharide (teichoic acid) preparations (8)

The keys for the identification of *S epidermidis sensu stricto* (designated *S epidermidis* in the following) and *S saprophyticus* were as follow

Simplified scheme (2)

	S epidermidis	*S saprophyticus*
Mannitol, acid (aerobically)	—	+
Phosphatase	+	—
Arginine dihydrolase	+	—
Subcommittee (13)		
*Sucrose acid (aerobically)	+	+
Trehalose, acid (aerobically)	—	+
Mannitol, acid (aerobically)	—	+
Phosphatase	+	—
Novobiocin	S	R

Sucrose is included to separate *S cohnii* from *S saprophyticus*.
S = sensitive R = resistant

Strains not identified as *S epidermidis*, *S saprophyticus* or *S cohnii* were tested on 5 per cent human blood agar plates by measuring colony diameter and zone of haemolysis after 24 48, and 72 h incubation (5) The size of the colony was found to be of no help in the classification and the results has not been included. Strains producing zone of haemolysis ≥2.5 mm out from the culture streak were identified as *S haemolyticus*. Remaining strains were tentatively identified after the abbreviated scheme of *Kloos & Schleifer* (5) using the criteria of the *Subcommittee* as well as haemolysis

RESULTS

Urinary Strains

The classification of the 128 urinary strains by the simplified scheme and by the *Subcommittee* scheme is shown in Table 1 Six strains were designated *S saprophyticus?* because they were ± instead of — in their phosphatase reactions. This usually occurred in the fluid medium. Eleven strains were designated *S saprophyticus?* because the trehalose test

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The type of wall teichoic acid was determined by double diffusion in agar using reference systems consisting of rabbit immune sera and polysaccharide (teichoic acid) preparations (8)

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Arginine dihydrolase	+	—
Subcommittee (13)		
*Sucrose acid (aerobically)	+	+
Trehalose, acid (aerobically)	—	+
Mannitol acid (aerobically)	—	+
Phosphatase	+	—
Novobiocin	S	R

Sucrose is included to separate *S cohnii* from *S saprophyticus*.
S = sensitive, R = resistant

Strains not identified as *S epidermidis S saprophyticus* or *S cohnii* were tested on 5 per cent human blood agar plates by measuring colony diameter and zone of haemolysis after 24 48, and 72 h incubation (5) The size of the colony was found to be of no help in the classification and the results have not been included. Strains producing zone of haemolysis ≥2.5 mm out from the culture streak were identified as *S haemolyticus*. Remaining strains were tentatively identified after the abbreviated scheme of *Kloos & S hleifer* (5) using the criteria of the *Subcommittee* as well as haemolysis.

RESULTS

Urinary Strains

The classification of the 128 urinary strains by the simplified scheme and by the *Subcommittee* scheme is shown in Table 1 Six strains were designated *S saprophyticus?* because they were ± instead of — in their phosphatase reactions. This usually occurred in the fluid medium. Eleven strains were designated *S saprophyticus?* because the trehalose test

was — or ± rather than + Three *S cohnii?* strains produced ± rather than—phosphatase reactions. These 20 strains all contained the typical AβC polysaccharide (2) and were beyond doubt, true *S saprophyticus* or *S cohnii* strains.

Six of the seven strains designated *S epidermidis?* by the simplified scheme, because of doubtful phosphatase reactions, were definitely shown by the criteria of the *Subcommittee* not to be *S epidermidis* and remained unclassified.

TABLE 1 *Classification of 128 Urinary Strains by the Simplified Scheme and the* Subcommittee *Scheme*

	Simplified scheme	*Subcommittee* scheme	
S epiderm	31	28	29
S epiderm.?	7	1	
S saprophyt	49	28	45
S saprophyt ?	6	17	
S cohnii		6	9
S cohnii?		3	
NC	35	45	

NC = Not Classified.

TABLE 2 *Tentative Classification of 45 Strains NC by the* Subcommittee *Scheme Adding Haemolysis to the* Subcommittee *Criteria*

**S haemolyt*	6
S hominis	11
S capitis	2
S simulans/ S warneri?	19
NC	7

* One strain mannitol-negative.
NC = Not Classified.

Disregarding the doubtful phosphatase and trehalose reactions, 29 strains could definitely be classified as *S epidermidis* 45 as *S saprophyticus* and nine as *S cohnii* the latter being distinguishable from *S saprophyticus* in its inability to attack sucrose. By the *Subcommittee* criteria as many as 45 strains (35 per cent) remained unclassified. When haemolysis was tested, the majority of these strains could be tentatively identified by means of the abbreviated scheme of *Kloos & Schleifer* (5) (Table 2) The six *S haemolyticus* strains seem to be correctly classified due to their strong haemolysis, and the characteristic biochemical pattern of *S hominis* should ensure the latter diagnosis. A correct classification of the 19 strains designated *S simulans* or *S warneri* was not possible by the criteria used

An attempt was made to evaluate the significance of the different species in urinary tract infection *S saprophyticus* and *S cohnii* were always present in significant numbers, whereas the other species/groups were found both in significant and in insignificant bacteriurias, the latter indicating contamination.

Strains from Routine Blood Cultures and Pus

The classification of 47 strains isolated in the diagnostic laboratory from blood cultures and pus samples is shown in Table 3

TABLE 3 *Classification of 47 Strains from Routine Blood Cultures and Pus*

	Simplified scheme	*Subcommittee* scheme
S epiderm	21	24
S saprophyt	1	
S saprophyt ?	1	
NC	24	23

NC = Not Classified

Forty strains gave identical results by the two classification schemes. Two strains classified as *S saprophyticus* by the simplified scheme were unclassified by the *Subcommittee* scheme. Both strains were sensitive to novobiocin Four strains were unclassified by the simplified scheme because the arginine dihydrolase reaction was negative These strains were definitely *S epidermidis* by the *Subcommittee* scheme, three of them contain-

ing poly Bz, the characteristic *S epidermidis* teichoic acid. One strain, classified as *S epidermidis* by the simplified scheme, contained poly Bz, but was recorded as unclassified by the *Subcommittee* scheme because the trehalose test was positive. When haemolysis was tested, 17 out of the 23 strains unclassified by the *Subcommittee* scheme were tentatively classified (Table 5). Seven strains belonged to *S haemolyticus* and 6 to *S hominis*.

In 19 cases the isolated organism was estimated to be the cause of infection. The different species/groups were represented both in cases of probable significance and in cases where contamination was more likely. This indicates that species other than *S epidermidis* may cause septicaemia and purulent infections. It was, however, noteworthy that in the three cases in which repeated isolations had shown the same strain, this was always *S epidermidis*.

TABLE 4. *Classification of 23 American and Canadian Strains from Blood Cultures and Pus (3 Strains)*

	Simplified scheme	*S bcommittee* scheme
S pidermi	14	17
NC	9	6

NC Not Classified

TABLE 5. *Tentative Classification of 29 Strains NC by the Subcommittee Scheme (Table 3 and Table 4) Adding Haemolysis to the Subcommittee Criteria*

	NC Strains	
	Table 3	Table 4
S haemolyt	7	
S hominis	6	2
S pis	1	
S simulans		
S warneri	3	1
NC	6	3

Four strains mannitol-negative
NC = Not Classified

TABLE 6. *Evaluation of Three Phosphatase Method in Total Material*

Phosphatase methods 1	2	3	Classification by the *Subcommittee* scheme	No. of strains	
+	+	+	*S pidermi*	69	74
			NC	5	
—	—	—	*S saprophyt*	40	84
			S cohnii	9	
			NC	35	
—	±	—	*S saprophyt*	3	31
			NC	28	
Other patterns			*S piderm.*	1	9
			S saprophyt	2	
			NC	6	

1 = *King* (3) 2 = *Pennock & Huddy* (9) 3 = *Satta et al.* (10)
NC = Not Classified.

American and Canadian Strains from Blood Cultures and Pus

Of the 23 American and Canadian strains isolated mainly from blood cultures in patients with endocarditis, 20 were identically classified by the two schemes (Table 4). Three strains had negative arginine dihydrolase tests by the simplified scheme and were recorded as unclassified. They were typical *S epidermidis* strains by the *Subcommittee* scheme, containing poly Bz. Of the six strains unclassified by the *Subcommittee* scheme, two were clearly *S hominis* (Table 5). Out of 20 strains isolated from blood cultures in patients with endocarditis and septicaemia, 16 were *S epidermidis*, one *S hominis*, one was possibly either *S simulans* or *S warneri*, and two strains were unclassified.

Evaluation of the Phosphatase Test

The results of the three phosphatase methods performed in the total material are shown in Table 6. In 158 strain, all the three methods gave clear-cut + or — reactions, consistent with the classification scheme for *S epidermidis* and *S saprophyticus/S cohnii*. With the exception of one *S epidermidis* strain, which was ± in the fluid medium but + with the other two methods, none of the

TABLE 7 *Content of Precipitinogens (Teichoic Acids) in Total Material*

	AαAβ	Bα	AβC	C	Others	NT	
S saprophyt	1		43		1		45
S cohnii			8		1		9
S epiderm		55	2	1	6	6	70
S hominis		1	1	4	3	10	19
S haemolyt			1	7	1	4	13
S capitis				1		2	3
S simulans/ S warneri?	1	1		7	2	12	23
NC		6		4	1	5	16
	2	63	55	24	15	39	198

Of the 15 strains grouped under "Others" 13 contained poly C in combination with other polysaccharides No strain contained protein A. NT = Not Typable. NC = Not Classified.

methods failed to produce positive results. The discrepancies were due to ± reactions and it was quite obvious that false positive ± *reactions occurred mainly in the fluid medium*. Three *S saprophyticus* strains were definitely false positive by this method whereas there is some doubt concerning the 28 unclassified strains The *Satta* method seemed to produce false positive results in a few strains. This occurred in only one instance by the *King* method.

Teichoic Acids

The results of the serologic investigation on teichoic acids are shown in Table 7 Eighty per cent of the strains were typable by the reference systems used *S saprophyticus* and *S cohnii* contain the same type of teichoic acid, there being a very close correlation between the presence of this teichoic acid (poly AβC) and the two species. Also the correlation between *S epidermidis* and poly Bα is very good. The six *S epidermidis* strains in which no teichoic acid was demonstrated as well as the six strains containing poly Bα but unclassified biochemically may be variants of *S epidermidis* Poly C alone is present relatively often in the remaining species/groups although the majority of the strains were untypable. No strain contained protein A.

DISCUSSION

The phosphatase reaction is a key criterion in the classification of *Micrococcaceae* The sensitivity of the classic method on solid medium (3) has been questioned (1) Therefore, one aim of the present investigation has been to compare *King's* method with the phosphatase reaction in fluid medium (9) and a method (A) developed by *Satta et al.* (10) All three reactions were reliable in producing positive results. The fluid medium, however produced a considerable number of non specific ± reactions. This must be taken into account when the fluid medium is used. *As a consequence* we would prefer using one of the two reactions on solid medium.

Although the two schemes for the classification of coagulase negative staphylococci here tested agreed in the vast majority of the strains, the comparison was in favour of the Subcommittee scheme. The disadvantage of a larger number of criteria was more than compensated for by the increased reliability of the latter scheme. In the simplified scheme a doubtful phosphatase reaction automatically created diagnostic problems, and, in addition, the arginine *dihydrolase test failed* in some instances. The additional criteria of the *Subcommittee* usually ascertained the diagnosis, even if the phosphatase test was equivocal In our hands, the trehalose test

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The Subcommittee scheme identifies only *S. epidermidis*, *S saprophyticus* and *S cohnii*. These are undoubtedly the most important coagulase-negative staphylococci in human infections, but the report of *Nord et al.* (7) as well as the present findings, indicate that other species may also be pathogenic, and even cause serious infections. In the present investigation as many as 35 per cent of the urinary strains could not be classified by the Subcommittee scheme. When the haemolytic activity on human blood agar plates was added to the criteria, a tentative identification according to the abbreviated scheme of *Kloos & Schleifer* (5) was possible for the majority of the non-classified strains. *S haemolyticus* and *S hominis* apparently were reliably identified, whereas *S simulans* and *S warneri* could not be separated.

In the diagnostic laboratory *S saprophyticus* (and *S cohnii*) can be identified very simply by means of the novobiocin test. To identify *S epidermidis* and other coagulase negative staphylococci, micrococci first have to be excluded by the glucose fermentation test, and then several criteria have to be tested. It is desirable that the diagnostic laboratories that have the capacity collect information on the distribution and clinical significance of all coagulase negative staphylococci, and that simple diagnostic criteria are agreed upon. Identification on a taxonomic level is not possible in the diagnostic laboratory.

In accordance with earlier reports (2) a very close correlation was demonstrated between the biochemical classification and the type of wall teichoic acid in *S epidermidis* and *S saprophyticus*. Some strains, however apparently were atypical in their biochemical properties, whereas others could contain minimal amounts of the typical teichoic acid or an atypical teichoic acid. With the criteria used in the present investigation, the only difference between *S saprophyticus* and *S cohnii* was the inability of the latter to attack sucrose. In contrast to *Schleifer & Kloos* (11) we found that eight out of nine urinary *S cohnii* strains contained the same teichoic acids as *S saprophyticus* i.e. β-N-acetylglucosaminyl ribitol teichoic acid and β-N-acetylglucosaminyl glycerol teichoic acid (poly AβC).

In the other species, no typical teichoic acid was demonstrated, although poly C was found quite frequently. In many of these strains no teichoic acid was demonstrated serologically. This might be due either to small amounts of wall teichoic acids (4) or to the strains containing a type of teichoic acid not represented in our reference systems.

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Acta path. microbiol. scand. Sect. B, 85 143–152, 1977

ANTIBIOTIC POLICY AND SPREAD OF *STAPHYLOCOCCUS AUREUS* STRAINS IN DANISH HOSPITALS, 1969–1974

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Rosendal, K., Jessen, O Bentzon, M W & Bülow P Antibiotic policy and spread of *Staphylococcus aureus* strains in Danish Hospitals, 1969–1974. Acta path. microbiol. scand. Sect. B, 85 143–152, 1977

In the period 1969–1974 a decreasing number of Danish hospital departments recorded epidemic occurrences of *Staphylococcus aureus* strains, and the local spread of strains was less extensive. Multiple-resistant strains of the 83 A complex were succeeded by type 94 strains, resistant to penicillin only. The level of methicillin resistance fell from 19 to 6 per cent. 1 per cent of methicillin resistant strains are now sensitive to streptomycin and/or tetracyclines; they do not represent few clones, but belong to a wide spectrum of phage types. The changes follow a reduction in the consumption of streptomycin and tetracyclines but not of methicillin or other penicillins. As contrast to the general reduction of combined resistance to streptomycin and tetracyclines, the strains in dermatological departments, where tetracyclines are commonly used, maintain a high degree of resistance to tetracyclines alone.

Key words: *Staphylococcus aureus*; antibiotic resistance; phage types; hospital infections; consumption of antibiotics

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Received 23.xi.76   Accepted 23.xi.76

Since 1969 Denmark has experienced a reduction of multiple-resistant *Staphylococcus aureus* strains, i.e. strains resistant to at least three antibiotics (5, 17). A similar development has been reported from other countries (2, 12, 14), where it happened earlier.

The present paper is an attempt to find out whether a correlation between the consumption of antibiotics and resistance to antibiotics can be found in the Danish material. During the period of increasing multiple-resistance, two studies from Danish hospitals suggested a connection with the use of penicillins and a series of other antibiotics (18), particularly with the consumption of tetracyclines and streptomycin (3).

Another factor, however, viz. hygienic measures, could also influence the spread of certain strains. We have tried to elucidate this problem by registrering identical strains with-

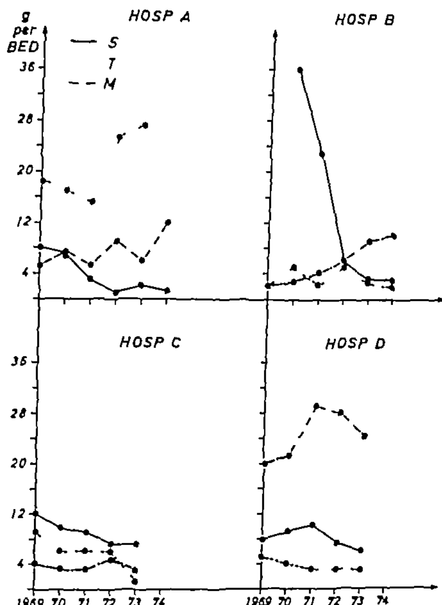


Fig. 1. Consumption of antibiotics in four Danish hospitals. S = streptomycin. T = tetracyclines. M = methicillin.

in various hospital departments in the years 1969 and 1974. This investigation might also reveal whether strains resistant to penicillin only were actually able to spread in the existing hospital environment, and probably characterize strains capable of producing an epidemic.

MATERIAL AND METHODS

Data were obtained from the central registration of *Staphylococcus aureus* strains (Statens Seruminstitut) isolated in Danish hospitals in the years 1969–1974 (17) and from the septicaemia material (5).

As an account of the antibiotics used throughout the whole country also involves veterinary medicine, it was necessary to seek information from the pharmacies of four big hospitals: two from the metropolitan area, hospitals A and B, and two provincial hospitals, C and D.

Epidemic strains were defined as strains of identical phage type and resistance pattern that occurred with a frequency of at least 5 per cent in hospital departments which in 1969 and 1974 had forwarded strains from at least 100 persons.

The bacteriological methods were given in a previous paper (17).

For an explanation of the nomenclature see Table 1.

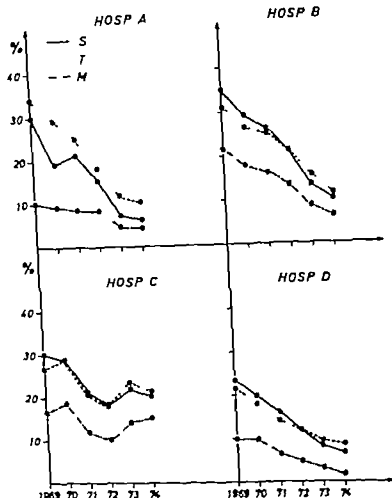


Fig 2 Antibiotic resistance of *S aureus* strains isolated in four Danish hospitals. S = streptomycin, T = tetracyclines, M = methicillin.

RESULTS

Antibiotic Policy and Changes in the Staphylococcal Flora

Data from four hospitals. An account of the consumption of antibiotics dispensed in all forms was obtained from four hospitals with a total of 4,800 beds—about 16 per cent of the hospital capacity of the country. A general trend was a high consumption of penicillinase-sensitive penicillins, including broad spectrum penicillins. During the period studied, this consumption rose by about 70 per cent. Another general characteristic was that the use of chloramphenicol (C) cephalosporins and erythromycin (E) was so restricted in comparison with the use of other antibiotics that no major selective role can be ascribed to these antibiotics.

For our present purpose the main interest centered on the use of streptomycin (S) tetracyclines (T) and methicillin (M). The consumption of these antibiotics per hospital bed is illustrated in Fig. 1. For the year 1974 data from two of the hospitals were not available.

TABLE 1 *Antibiotic Resistance among S aureus Strains Isolated from Danish Dermatological Departments 1973*

Antibiotic resistance pattern	All dermatological departments	Dept. I	Dept. II	Total material 1973
PT and T	25 %	17 %	35 %	5 %
PST + *	6 %	9 %	7 %	10 %
PSTM + *	1 %	2 %	0	7 %
Total number of strains	771	128	14*	20,254

P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol E = erythromycin M = methicillin.
*+ = ± resistance to C and/or E.
From departments *I* and *II* only one strain of each phage type (pattern) within each antibiotic resistance pattern is included.

In all four hospitals the use of S was reduced considerably in hospital B dramatically so

The consumption of T also declined except during the years 1972 and 1973 in hospital A.

The use of M was double or more than that in hospitals A and B rather constant in hospital C and—by comparison—very high in hospital D

During the period investigated *Staphylococcus aureus* strains from 37 758 patients from the four hospitals were examined these strains are considered representative of the local staphylococcal flora (Fig 2)

The general tendency in all four hospitals was a fall in the proportion of strains resistant to S T and M In hospital C the tendency was interrupted in 1973 a detailed investigation of the strains attributed this increase to a temporary rise in the level of hospital infections due to an epidemic multiple-resistant strain.

The decrease in the number of M resistant strains at all of the hospitals occurred despite an increasing or constant consumption of M the lowest level of M resistance was obtained at hospital D which had by far the largest consumption of M

The large consumption of T at hospital A was not accompanied by a high level of T resistance, and the small peak of multiple-resistant strains at hospital C in 1973 was not preceded by any increased use of antibiotics

Data from dermatological departments. From the investigation of the central staphylococcal registration it appeared that generalizations for hospitals as a whole did not apply to dermatological departments. These departments maintained a considerable use of T also topically applied whereas S was used in very small amounts.

In Table 1 unselected strains from Danish hospitals 1973 have been compared with strains from all dermatological departments.

The outstanding features in the dermatological departments were the large number of strains resistant to T despite sensitivity to S, and the small number of M resistant strains. All of the latter were also resistant to penicillin (P) S and T their sensitivity patterns and phage types corresponded to those of common hospital strains. They are assumed to have been introduced from other hospital departments, but the low figures indicate their inability to establish themselves as epidemic strains in the dermatological departments.

On the other hand, the strikingly large number of T resistant strains sensitive to S does not correspond to the staphylococcal flora, neither in hospitals nor in the community Among septicaemia strains from 1969–1974 the percentage frequency of

strains with this antibiotic pattern was 3 (hospital) and 4 (community).

Apart from the rare multi-step mutation, two possible explanations remain: the spread of plasmids, determining T resistance, or a local spread of a limited number of strains with the particular antibiotic resistance mentioned.

TABLE 2. Occurrence of Methicillin Resistant Strains Sensitive to S and/or T

Year	Total number of methicillin resistant strains	Per cent sensitive to S and/or T
1969	2155	2.1
1970	2193	2.0
1971	1723	3.0
1972	1634	4.2
1973	1130	5.1
1974	869	12.1
Phage group/complex		
52, 52A, 80, 81 complex	90	34
Group I	60	47
Group II	57	47
Group III	644	10
83A complex	8433	2
Others	100	34
NT (non-typable)	518	17

The latter possibility has been tested by a separate detailed study of the two largest dermatological departments in the country—here named I and II. In order to exclude the influence of cross infection, the material of strains from these departments was reduced to comprise only one representative of isolates with identical phage type and resistance pattern. From Table 1 it is seen that the unusual resistance pattern is found even among these epidemiologically unrelated strains.

Methicillin resistance. The proportion of M resistant strains has been reduced from a strikingly high level of 19 per cent and even 40 per cent in hospital acquired bacteraemia (5) in 1969 to 6 per cent in the general material in 1974 (17).

The majority of M resistant strains belonged to a single or few phage type complexes and were resistant also to S and T.

A large material as the present, however, shows that M resistance is also found in strains sensitive to S and/or T (i.e. "abnormal" M resistance pattern) (Table 2) with a frequency that increases to 12 per cent in 1974.

The lower part of Table 2 shows that these "abnormal" M resistant strains do not represent a few clones. It gives the percentage of strains with "abnormal" M resistance patterns within different phage groups/complexes in the total material of 9 704 M resistant strains. With the exception of the epidemic strains of the 83 A complex, they seem to occur frequently in all phage groups. Thus, M resistance is neither strictly limited to strains resistant to S and T nor to single phage types, but occurs in a wide spectrum of strains, which until now has been overshadowed by a few strains with epidemic properties.

TABLE 3. Number of Epidemic Strains* per Department§

Number of strains	Number of departments 1969	1974
0	2	12
1	16	8
2	10	7
3	7	1
4	1	0
Total	38	28

* Strains isolated with a frequency of at least 5 per cent.

§ From which specimens from at least 100 persons were forwarded.

Epidemic Strains 1969 and 1974

Recognition of epidemic strains. An attempt was made to estimate the extent of cross infection by registrering all strains of identical phage types and resistance patterns occurring with a frequency of at least 5 per cent in hospital departments that in 1969 and

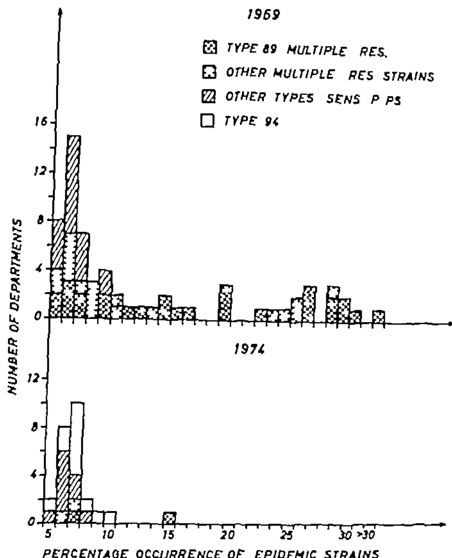


Fig 3 Percentage occurrence of "epidemic types" in various departments.

1974 had forwarded strains from at least 100 persons.

From Table 3 it is seen that the material from 1974 differs from that of 1969 in several respects. Fewer departments forwarded 100 specimens, and fewer strains were represented by at least 5 per cent, despite an increase of about 2000 in the total number of strains examined.

Moreover the percentages of strains of "epidemic occurrence ($\geqq$ 5 per cent) were considerably lower (Fig 3) In 1969 the epidemic strains in 25 departments exceeded 10 per cent of the total number forwarded, *whereas in 1974 only one department could* register this frequency

In 1969 epidemic multiple-resistant strains were found in 45 departments, as against 4 in 1974 The epidemic strains were M resistant—and always resistant to S and T as well —in 33 cases in 1969 *but only in 4 cases* in 1974

Phage types and antibiotic resistance of epidemic strains. From Table 4 it is seen that the changes also concern the phage type and *the antibiotic pattern of the epidemic strains.*

The dominating epidemic strains of the 83 A complex have lost their importance the number of departments where multiple-resistant strains of this category are epidemic, have been reduced from 43 to 4 those resistant to P only have disappeared. Also strains

TABLE 4 *Phage Type and Antibiotic Resistance of Epidemic Strains*

Phage group/complex	Phage types	Antibiotic sensitivity	Number of departments 1969	1974
Group I	52/52A/80	Sensitive	2	0
	52/52A/80	PS	4	0
	52/52A/80	PST	1	0
	29 29/52 52/52A; 52/79	P	0	6
Group II	3A 3C/55/71	Sensitive	2	0
	3A 3A/3C	P	6	3
Group III	75A = 6/47/53/81/83A/84/85/95/89	P	2	0
83A complex	84/85/93/89	P	3	0
	84/85/93/89 84 85 89	PST +	43	4
Miscellaneous	94	P		12
Number of epidemic strains			63	25

For an explanation of the nomenclature, see Table 1

of the 52 52A, 80 81 complex and group III strains have vanished, whereas in a few departments group II strains still remain. However in 1974 the most common epidemic strains were P resistant staphylococci of type 94 and to a lesser extent group I strains, in 3 cases belonging to phage type 29/52.

In 1969 four departments harboured more than 5 per cent fully sensitive strains of a single phage type. Since these strains in the country as a whole accounted for only 1–2 per cent each, the relative frequency in single departments was probably due to a certain spread of the strains in question.

Thus, the epidemic situation has undergone changes, and the spread of identical strains has decreased considerably

Phage type 94 Phage type 94 is a new strain which has recently gained a foothold in the hospital environment in Denmark as well as in U.S.A. (16)

Phage 94 (1) was not included in the basic set of typing phages until 1973 but by retyping freeze-dried septicaemia strains the first appearance of type 94 could be established in 1969 However it is highly improbable that it occurred as an epidemic strain in the present material in 1969 If so, it would have appeared as non-typable, and such strains were not recorded with a frequency of 5 per cent in any department in 1969

In 12 departments included in Table 4 P resistant type 94 strains account for an average of 7 to 8 per cent of the infections. In the total material from Danish hospitals from the years 1973 and 1974 it amounts to 4 and 5 per cent of the strains, respectively

No particular source of the isolates can be pointed out. 4 to 6 per cent of the strains from abscesses, boils, cicatrices, wounds, tracheal secretions and sputum belonged to type 94 and 2 per cent of those from urine.

In septicaemia cases, type 94 was isolated in 5 per cent of the cases in 1974 but in 1975 with a frequency of 10 per cent. The mortality rate of these infections is about 39 which is equal to that of other strains with the same antibiotic resistance.

The antibiotic resistance of type 94 is seen from Table 5

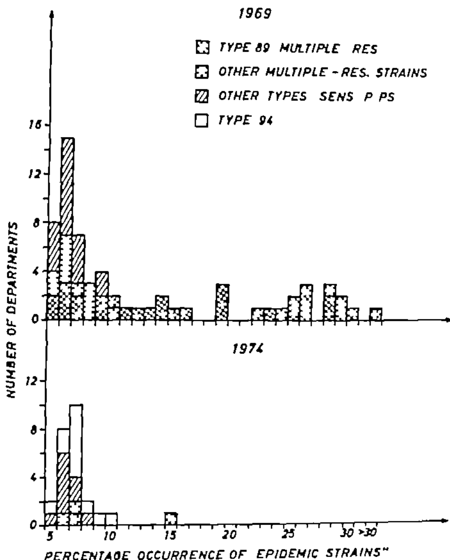


Fig 3 Percentage occurrence of "epidemic types' in various departments.

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From Table 3 it is seen that the material from 1974 differs from that of 1969 in several respects. Fewer departments forwarded 100 specimens and fewer strains were represented by at least 5 per cent despite an increase of about 2000 in the total number of strains examined

Moreover the percentages of strains of epidemic" occurrence (≧ 5 per cent) were considerably lower (Fig 3) In 1969 the epidemic strains in 25 departments exceeded 10 per cent of the total number forwarded, whereas in 1974 only one department could register this frequency

In 1969 epidemic multiple-resistant strains were found in 45 departments, as against 4 in 1974 The epidemic strains were M resistant—and always resistant to S and T as well —in 33 cases in 1969 but only in 4 cases in 1974

Phage types and antibiotic resistance of epidemic strains From Table 4 it is seen that the changes also concern the phage type and the antibiotic pattern of the epidemic strains.

The dominating epidemic strains of the 83 A complex have lost their importance the number of departments where multiple-resistant strains of this category are epidemic, have been reduced from 43 to 4 those resistant to P only have disappeared Also strains

TABLE 4 *Phage Types and Antibiotic Resistance of "Epidemic Strains"*

Phage group/complex	Phage types	Antibiotic sensitivity	Number of departments 1969	1974
Group I	52/52A/80	Sensitive	2	0
	52/52A/80	PS	4	0
	52/52A/80	PST	1	0
	29; 29/52 52/52A; 52/79	P	0	6
Group II	3A 3C/55/71	Sensitive	2	0
	3A 3A/3C	P	6	3
Group III	73A 6/47/53/81/83A/84/85/95/89	P	2	0
83A complex	84/85/93/89	P	3	0
	84/85/93/89 84 85; 89	PST +	43	4
Miscellaneous	94	P		12
Number of epidemic strains			63	25

For an explanation of the nomenclature, see Table 1

of the 52, 52A, 80 81 complex and group III strains have vanished, whereas in a few departments group II strains still remain. However in 1974 the most common epidemic strains were P resistant staphylococci of type 94 and to a lesser extent group I strains, in 3 cases belonging to phage type 29/52.

In 1969 four departments harboured more than 5 per cent fully sensitive strains of a single phage type. Since these strains in the country as a whole accounted for only 1-2 per cent each, the relative frequency in single departments was probably due to a certain spread of the strains in question.

Thus, the epidemic situation has undergone changes, and the spread of identical strains has decreased considerably

Phage type 94 Phage type 94 is a new strain which has recently gained a foothold in the hospital environment in Denmark as well as in U.S.A. (16)

Phage 94 (1) was not included in the basic set of typing phages until 1973 but by retyping freeze-dried septicaemia strains the *first* appearance of type 94 could be established in 1969 However it is highly improbable that it occurred as an epidemic strain in the present material in 1969 If so, it would have appeared as non-typable, and such strains were not recorded with a frequency of 5 per cent in any department in 1969

In 12 departments included in Table 4 P resistant type 94 strains account for an average of 7 to 8 per cent of the infections. In the total material from Danish hospitals from the years 1973 and 1974 it amounts to 4 and 5 per cent of the strains, respectively

No particular source of the isolates can be pointed out. 4 to 6 per cent of the strains from abscesses, boils, cicatrices, wounds, tracheal secretions and sputum belonged to type 94 and 2 per cent of those from urine.

In septicaemia cases, type 94 was isolated in 5 per cent of the cases in 1974 but in 1975 with a frequency of 10 per cent. The mortality rate of these infections is about 39 which is equal to that of other strains with the same antibiotic resistance.

The antibiotic resistance of type 94 is seen from Table 5

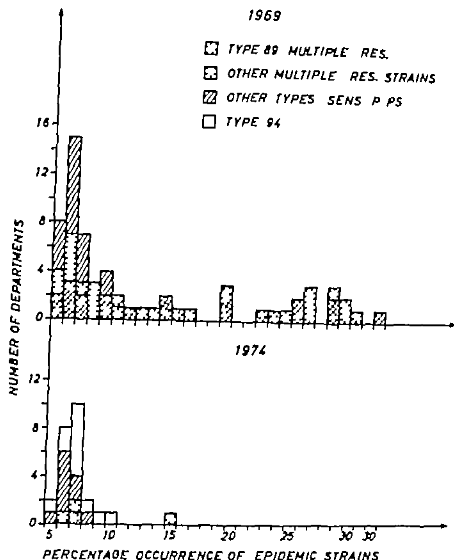


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stant strains, either in this country or in other countries (6, 12, 14) could not be correlated with the consumption of M and it seems probable that the M resistant multiple-resistant strains, having a more fragile structure (4) than the corresponding M sensitive ones, require a protective pressure from S and T in order to maintain a dominating position. This view is supported by an observation from the years 1972–1974 with diminishing but still continuing use of S and T among 60,000 strains the number of strains resistant to S and T but sensitive to M have been stationary whereas the corresponding strains resistant to M are still in retreat.

In Denmark, all the M resistant strains causing epidemics belonged to the 83 A complex and were resistant to S and T as well. In so far the observations seem to support *Lacey's* mono-clone theory (7 9) according to which the M resistant strains represent one or a few clones of staphylococci. However M resistant strains, sensitive to S and/or T are isolated with increasing frequency. Furthermore they belong to many different phage types (Table 2) and none of these strains play any epidemiological role. Therefore, it is possible that the mono-or oligo-clone theory could be modified to imply that out of a variety of M resistant strains, only a few clones with special characteristics have until now shown a capability to spread to any greater extent.

In our experience the use of ordinary penicillins (13) and newer" antibiotics, *e.g.* cephalosporins (2, 11 15 19) cannot be connected with the decline of the M resistant strains, since the penicillins are still being used in increasing amounts, and the consumption of the other drugs is negligible.

Even though environmental changes influence the emergence and disappearance of epidemic strains, it must be admitted that the obvious differences in contagiosity between staphylococcal strains largely remain unexplained. From Fig. 3 it is seen that in 1969 the multiple-resistant type 89 was far more frequently isolated than any other multiple resistant type and in 1974 type 94 resistant to P only was dominating but spreading at a slower rate than type 89 previously. As fundamental information on the mechanisms and genetics of contagiosity is still lacking it is still not clear why these strains are superior to other types with the same antibiotic pattern.

Studies of changing epidemic strains must be continued, as from an epidemiological point of view the present 20-year period is too short to allow any definite conclusions to be made concerning the influences governing the fluctuation of the staphylococcal flora.

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TABLE 5 *Antibiotic Resistance of 1964 Strains of Phage Type 94*

Resistant to		Per cent
P		93
S		3
T		11
C		1
E		1
M		2
None		7
P	Sensitive to	80
PT or T	other antibiotics	9

TABLE 6 *Septicaemia Strains 1969–1974*

	Type 89	Type 94
Multiple resistant	96 %	4 %
TW—	89 %	3 %
Hg +	94 %	3 %
Total number	407	79

TW— = do not produce the 'Tween-80-splitting enzyme.
Hg + = resistant to mercuric chloride.

In Table 6 some characteristics of type 94 and of the previous dominating strain type 89 are given

DISCUSSION

The changing resistance patterns in staphylococci have in several but far from in all cases studied shown a time relation with the consumption of antibiotics. Danish studies during the period of increasing multiple resistance have suggested the use of penicillins and possibly a series of other antibiotics (18) or in particular the consumption of S and T (3) as possible determinants for the development. Several other examples—most of them concerning single hospitals for a short period of time—have been discussed recently by *Plorde & Sherris* (14)

In the present material the examples from dermatological departments suggest a connection between the antibiotic policy of a department and the resistance pattern emerging from the spread of plasmids, determining T resistance, among epidemiologically independent strains.

On the other hand the staphylococcal flora of the four selected hospitals was influenced by the spread of identical strains, of which a declining number were multiple resistant. A diminished spread caused for example, by an improved hospital hygiene has actually been suggested as a possible explanation of the general reduction of the number of multiple resistant strains (10 19) In the present case, however improved hygiene can hardly be the sole factor since a spread, admittedly reduced, of single phage types is still evident, and with the important difference that the predominant epidemic strains now are resistant to P only

In this development, a change of the antibiotic policy might be an important determinant, provided less resistant strains are superior to multiple-resistant ones under the altered circumstances due to the restricted use of some antibiotics This may be the case if it is generally true that multiple resistant strains have a slower growth-rate than more sensitive strains (8)

The minor differences in the antibiotic policy at the four hospitals are not clearly reflected in the local antibiotic resistance situation, and it must be stressed that observations from single hospitals or departments over a short period of time—whether reported here or in other studies—are conclusive to a very limited extent only However the general tendencies are considered typical for Danish hospitals, and consist of a reduction in the use of S and T but not of penicillins. In hospitals A and D this development is known to have started several years previously (3)

A country wide reduction of the selective pressure exerted by S and T may well have been a pre-requisite for the present changes, and may in particular have reduced the number of M resistant strains, of which the epidemic ones were resistant to S and T

It is a fact that the occurrence of M resi

Acta path. microbiol. scand. Sect. B, 85: 153–160, 1977

THE ULTRASTRUCTURE OF *LEISHMANIA TROPICA* IN SKIN LESIONS

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Hentzer, B. & Kobayasi, T. The ultrastructure of *Leishmania tropica* in skin lesions. Acta path. microbiol. scand. Sect. B, 85: 153–160, 1977.

The ultrastructure of the amastigote stage of *Leishmania tropica* has been studied. The present work reports on new observations on the fine structure of the basal body, prokinetosome and flagellum. The basal body is composed of a centriole-like structure and a transitional zone continuous with the proximal part of the flagellum. The centriole-like part consists of nine peripheral triplet tubules and an indistinct dense central core. The transitional zone consists of nine peripheral doublet tubules and two central cylinders. The centriole-like prokinetosome is present parallel and close to the basal body. The flagellum arises from the transitional zone of the basal body and has the classical axoneme (9+2) structure. However, at the tip of the flagellum the central tubules are replaced by one of the peripheral doublet tubules. The ultrastructural findings, as compared with previous studies of *Leishmania*, are discussed.

Key words: *Leishmania tropica*; leishmaniasis; protozoa; parasites.

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Received 28.ii.76 Accepted 27.xi.76

The genus *Leishmania* belongs to the family of *Trypanosomatidae*. The *Leishmania* flagellates exist in two different stages, the amastigote and the promastigote stage (7). The promastigote stage of *Leishmania tropica* (*L. tropica*) is transmitted to man by bites of sandfly of the phlebotomus family. The protozoon produces skin lesions only (oriental sores, 4, 17).

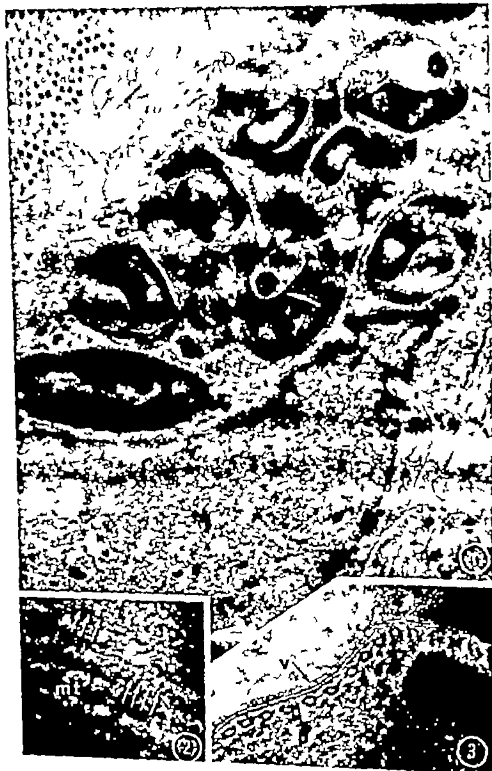
The first study on the ultrastructure of *L. tropica* was reported by *Löfgren* in 1950 (9). However, that author examined only the promastigote stage. The amastigote stage has been studied by *Pham et al.* (13), *Gardener* (6) and *Bourlond-Reinert & Nicolay* (2). The morphology of *L. donovani* and *L. mexicana* has been studied by *Rudzinska et al.* (18), *Sanyal & Sen Gupta* (19) as well as by *Creemers & Jadin* (5).

The present study forms the basis of further studies on the influence of antiprotozoal drugs on the morphology of *L. tropica* (8). Comparing our results with previous studies, we found that the fine structure of the basal body and the flagellum of *L. tropica* was insufficiently described. The aim of the present work was, therefore, to study these structures in detail.

MATERIAL AND METHODS

Punch skin biopsies were taken from nodular lesions of a 74-year-old male suffering from oriental sores. The specimens were fixed in a 6 per cent solution of glutaraldehyde in veronal acetate buffer pH 7.4 with 7.5 per cent sucrose at 4° C overnight. The

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mt
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(2)
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tissue blocks were then postfixed in 1 per cent osmic acid in veronal acetate buffer pH 7 4 at 4 C for one hour After washing in the same buffer the blocks were dehydrated in a series of graded alcohols and embedded in Epon 812 by *Luft s* technique (10) Ultrathin sections were cut by an LKB ultramicrotome and stained with uranyl acetate and lead citrate The sections were studied by a Siemens electron microscope (Elmiskop 1 A) at 80 kV with a double condensor system.

RESULTS

Sections of more than 200 *L. tropica* protozoa were studied All protozoa were phagocytized by dermal macrophages and enclosed in phagocytic vacuoles (Fig 1) The shape of the parasite was ovoid with a maximum diameter and a length of 1µ and 2.5 µ respectively

The *pellicle* bounding the individual protozoa consisted of a 7 3 nm thick unit membrane and a palisade of microtubules (Fig 3) Each microtubule was 32 2 nm in diameter and had a 8 3 nm thick rim The microtubules ran parallel to each other at a distance of 16 6 nm. An average of 94 microtubules were counted in each parasite. The microtubules were linked to one another with 3.5 nm thick fibrils which were arranged perpendicular to the microtubules (Figs. 2 3) On longitudinal sections of the microtubules, the linking fibrils were parallel to each other at intervals of 21 6 nm (Fig 2)

The *flagellar pocket* could be seen at the posterior end of the cell as an invagination of the unit membrane of the pellicle (Figs. 4 5) and consisted of a dilated basal part, a narrow funnel like part and an opening (Fig 5) There were no microtubules present beneath the membrane in the pocket wall Concentric lamellar electron dense material was found immediately beneath the unit membrane of the funnel like part and the opening of the pocket (Figs 14 15) The phagocytic vacuole communicated with the pocket through the opening (Fig 5) The fine granular material, myelin figures and double membrane-bounded bodies inside the flagellar pocket and the phagocytic vacuole were identical (Figs. 5 6)

The *basal body* consisted of two parts, i e a centriole-like structure and a transitional zone continuous with the proximal part of the flagellum (Fig 4) The centriole-like part was cylindrical, with a length of 125 nm and a diameter of 200 nm (Figs. 4 7 8) The wall consisted of nine triplet tubules, each forming an angle of 30–45 degrees with the tangent to the circumference (Fig 9) The individual tubule was 25-29 nm across and had a rim of 5-8 nm. An indistinct dense core was seen in the centre. At the proximal part of the basal body an electron dense basal foot merged with the wall (Figs. 4 8) The two innermost A and B-tubules of the proximal part of the basal body triplet were continuous with the peripheral doublet tubules of the transitional zone. At the level of C-tubule termination there was a thin terminal plate (Figs. 4 8) just above the level of the bottom of the flagellar pocket. Near and parallel to the basal body a similar second aflagellate basal body called prokinosome, could occasionally be seen (Fig 7) The transitional zone was located between the terminal plate of the basal body and the axosome of the flagellum (Figs 4 7) At the centre of the transitional zone there were two indistinct parallel cylinders, (Figs. 4 11) each of which was 54 nm in diameter and 280 nm long. The cylinders terminated in the electron dense axosome (Fig 4) At the periphery

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Fig 1 A part of dermal macrophage which has phagocytized nine *L. tropica* protozoa is seen. × 20 000

Fig 2 The palisade of microtubules (mt) is shown in a longitudinal section. The linking fibrils can be seen between and perpendicular to the individual tubules, (arrows) × 120 000

Fig 3 The construction of the pellicle is shown in a cross section. The unit membrane of the pellicle is seen (arrow-p) above the microtubules with their linking fibrils. The unit membrane of the phagocytic vacuole (arrow-v) of the host cell covers most of the pellicle but is separated from this in the right side of the micrograph. The phagocytic vacuole (pv) is filled with a fine granular material. × 120 000


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nine doublet tubules were found (Figs. 9 10 11) Each pair consisted of one complete tubule with a diameter of 29 nm and a rim of 8 nm and one incomplete tubule gripping onto the wall of the complete tubule (Figs. 14 17) The centre of the incomplete tubule was frequently filled with dense material. From the complete tubule two small side-arms extended (Fig 17) The nine doublet tubules extended from the basal body to the flagellum

The flagellum extended through the flagellar pocket and stopped immediately outside the pocket opening (Fig 5) The proximal part of the flagellum was 250 nm in diameter but became thinner in the funnel like part of the pocket. At the pocket opening the diameter was reduced to half the size (Figs. 5 16) Two central tubules with a diameter of 25 nm and a rim of 5 nm extended from the axosome to the distal part of the flagellum (Figs 4 13 14 15) The axosome appeared as an electron-dense disc measuring 92 nm across and 25 nm in thickness (Figs. 4 12) Two planes, one through the axis of the cylinders of the trans tional zone and the other through the central tubules of the flagellum, met at a right angle. At the tip of the flagellum the central tubules were replaced by one of the peripheral doublet tubules which had been displaced towards the centre (Figs. 15 16)

The *kinetoplast* was located in the dilated end of the mitochondrion lying close to the basal body (Figs 4 7 8) The diameter of the disc shaped structure was 700 nm and the height 100 nm The kinetoplast consisted of a parallel array of lamellae lying parallel to the long axis of the cell. The average thickness of the individual lamella was 16 nm

In addit on to these structures which were specific for the protozoon also ordinary cell organelles were found. *L. tropica* had a single oblong mitochondrion with relatively few cristae The Golgi complex and the endoplasmic reticulum were seen only occasionally Free ribosomes were abundant (Fig 4) On an average the nucleus occupied one quarter of the cell (Fig 1) Both membrane bounded dense bodies and multivesicular bodies could be seen frequently There were small vacuoles in the vicinity of the flagellar pocket.

DISCUSSION

Many of our observations concerning the ultrastructure of *L tropica* apply to most species of *Leishmania* but some exceptions must be emphasized

It has been claimed that the pellicle consisted of two unit membranes and a palisade of microtubules (18) We found that the outermost unit membrane was derived from the phagocytic vacuole The observation of *Pham et al* (13) supports this view

The linking fibrils between the microtubules of the pellicle were mentioned by *Rudzinska et al* (18) in *L. donovani*. Such fibrils have not been described in *L. tropica* (2 6 13) Linking parallel fibrils, regularly arranged at right angles to the microtubules, have been reported in *Hypotrichomonas* (12)

The different species of *Leishmania* have various numbers of microtubules in their pel

Fig 4 The basal body (b) and the flagellum are cut longitudinally through the centres. The proximal part of the basal body can be seen close to the kinetoplast (k) The transitional zone with two central cylinders (c) is separated from the basal body by the terminal plate (t) The central tubules (ct) of the flagellum arise from the axosome (arrow a) The peripheral doublet tubules (pt) extend from the basal body through the flagellum. Many ribosomes (r) are seen in the cytoplasm of the protozoon. Cross sections of the flagellum at the levels of the broken lines with numbers 10 to 14 are shown in the various corresponding figures. × 40 000

Fig 5 The micrograph shows the flagellar pocket (fp) as well as the flagellum The unit membrane of the pocket (arrow-p) is continuous with the unit membrane of the pellicle. The unit membrane of the phagocytic vacuole (arrow-v) is separated from the pellicle Cross sections at the broken lines 15 and 16 are shown in Figs. 15 and 16 × 40,000.

Fig 6 The granular material in the flagellar pocket with double membrane-bounded particles (arrows) and myelin like figures (my) can be seen. × 60 000

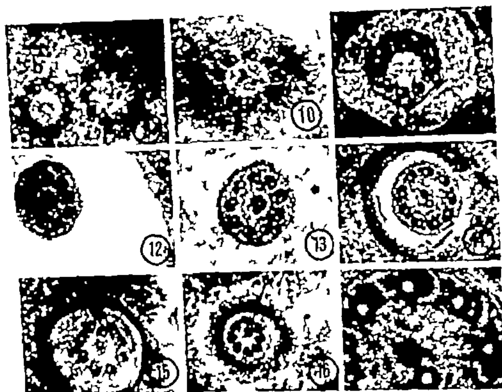


Fig 9 Cross section of the basal body (b) just below the C-tubule termination and of the prokinosome (pk) The wall of the prokinosome consists of triplet A B- and C-tubules. × 80,000

Fig 10 Cross section of the basal body t the level of the terminal plate The wall of the basal body consists of nine doublet tubules × 80,000.

Fig 11 Cross section of the transitional zone of the basal body shows two indistinct central cylinders arrow-c) and nine peripheral doublet tubules The unit membrane (arrow) of the flagellar pocket is also shown. × 80,000

Fig 12 Cross section of the axosome (arrow) × 80,000

Fig 13 Cross section at the base of the axoneme Two indistinct central tubules merge with the axosome × 80,000

Fig 14 Cross section of the flagellum in the funnel-like part of the flagellar pocket. Beneath the unit membrane of the pocket, concentric electron-dense lamellar material (arrow) is seen The flagellum consists of nine peripheral doublet tubules and two central tubules (axoneme structure 9+2) × 80,000.

Fig 15 Cross section of the distal part of the flagellum. One of the peripheral doublet tubules is displaced towards the centre (arrow) × 80,000

Fig 16 At the extreme end of the flagellum, the tw central tubules disappear and are replaced by one of the peripheral tubules (arrow) The diameter of the flagellum is reduced to half the size. × 80,000

Fig 17 An enlargement of Fig 14 which show part of the peripheral doublet tubules of the flagellum. Each pair consists of one complete and one incomplete tubule On the complete tubule tw side-arms are seen (arrow) × 240,000.

The flagellum of *L. tropica* possesses the classical axoneme structure (9+2) The present study stresses that the central tubules arising from the axosome do not extend to the tip of the flagellum. The replacement of central tubules by one of the





Fig 7 The basal body (b) and the aflagellate prokinosome (*pk*) *are located close to the kinetoplast* containing mitochondrion. Broken line 9 indicates the plane of the cross section shown in Fig. 9. × 80 000

Fig 8 Basal feet (arrows) are shown merging with the wall of the proximal end of the basal body Note the distinct terminal plate (t) appearing at the level of the C-tubule termination, × 80,000.

licle. In *L. mexicana* there are 130–200 (7) *L. donovania* has 80–120 (19) and *L trop-ica* 90 (2) *This latter result is in accordance* with our observations. The palisade of micro-tubules linked with fibrils probably supports the external body form No pellicle-associated structures, *i e* cytostome or posterior invagination of the type reported by *Pham et al* (13) and *Gardener* (6) in different species of *Leishmania* were seen in the present study

It is well known that the basal body and *the centriole are related structures (16) Basal* bodies occur at the base of both cilia and flagella (14) *Garnham* (7) claims that the basal body of *L mexicana* contains 9 doublet tubules However *Gardener* (6) and *Bourlond Reinert & Nicolay* (2) and our observations show that the body wall consists of nine triplet tubules. The appearance of a second aflagellate centriole like prokinosome close to the basal body is well-known in many pathogenic flagellates (23 14) In conventional centriole *replication a procentriole forms* at a right angle to the mature interphase centri*ole (15) However in L. tropica* the basal body and the prokinosome are always found *parallel* to each other

Root structures, *i.e* basal feet, have not been found in *L tropica* previously The basal foot is a common appendage of the basal body of most cilia and flagellae (14) No striated roots were found. The function of the basal feet is not known, but may be an anchoring of the basal body to the surrounding cytoplasm.

The transitional zone of the basal body has never been reported in *Leishmania* (2, 3 5 7 9 13 18 19) In the present study the basal body including the transitional zone is similar to the type II basal body described by *Pitelka* (14) with the exception of the central cylinders Type II basal bodies have been found in kinetoplastal flagellates (1 2?) The function of the transitional zone is *not clear but it does not seem to participate* in the movement of the flagellum (20) The *significance* of the central cylinders is also unknown

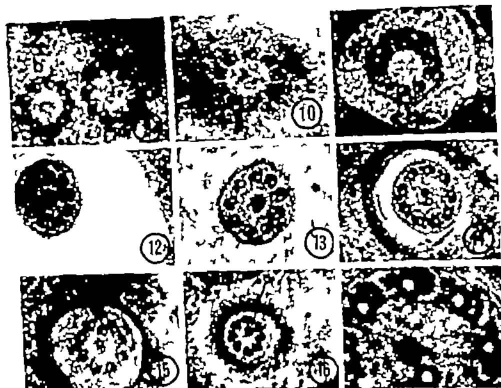


Fig 9 Cross section of the basal body (b) just below the C-tubule termination and of the prokinosome (pk) The wall of the prokinosome consists of triplet A- B- and C-tubules. × 80,000

Fig 10 Cross section of the basal body at the level of the terminal plate The wall of the basal body consists of nine doublet tubules. × 80,000

Fig 11 Cross section of the transitional zone of the basal body shows two indistinct central cylinders (arrow-c) and nine peripheral doublet tubules. The unit membrane (arrow) of the flagellar pocket is also shown × 80,000

Fig 12 Cross section of the axosome (arrow) × 80,000.

Fig 13 Cross section at the base of the axoneme Two indistinct central tubules merge with the axosome × 80 000

Fig 14 Cross section of the flagellum in the funnel-like part of the flagellar pocket. Beneath the unit membrane of the pocket, a concentric electron-dense lamellar material (arrow) is seen. The flagellum consists of nine peripheral doublet tubules and two central tubules (axoneme structure 9+2) × 80 000.

Fig 15 Cross section of the distal part of the flagellum. One of the peripheral doublet tubules is displaced towards the centre (arrow) × 80,000.

Fig 16 At the extreme end of the flagellum, the two central tubules disappear and are replaced by one of the peripheral tubules (arrow) The diameter of the flagellum is reduced to half the size. × 80,000

Fig 17 An enlargement of Fig 14 which shows part of the peripheral doublet tubules of the flagellum Each pair consists of one complete and one incomplete tubule. On the complete tubule two side arms are seen (arrow) × 240,000

The flagellum of *L. tropica* possesses the classical axoneme structure (9+2) The present study stresses that the central tubules arising from the axosome do not extend to the tip of the flagellum. The replacement of central tubules by one of the

peripheral doublet tubules has not been observed in *Leishmania* previously However a micrograph of *L. tropica* in a paper by *Bourlond Reinert & Nicolay* (2) shows the phenomenon though it is not mentioned

The kinetoplast is an organelle containing DNA (23) During replication the DNA material of the kinetoplast divides before the actual division of the nucleus takes place (7) Many antiprotozoal drugs specifically induce ultrastructural changes of the DNA kinetoplast of *Leishmania* (8 11 21) The significance of these findings must be based on a comprehensive study of normal flagellates.

The authors wish to thank Miss *Lise Fredebo* and Mrs *Birthe Brobeck* for their valuable technical assistance.

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Acta path. microbiol. scand. Sect. B, 85 161 166 1977

EFFECTS OF AMPICILLIN ON INTRACELLULAR LEVELS OF ADENOSINE TRIPHOSPHATE IN BACTERIAL CULTURES RELATED TO ANTIBIOTIC SUSCEPTIBILITY

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Thore, A., Nilsson, L. Höjer H Ansehn, S & Bröte, L. Effects of ampicillin on intracellular levels of adenosine triphosphate in bacterial cultures related to antibiotic susceptibility Acta path. microbiol. scand. Sect. B, 85: 161–166 1977

Short time effects of ampicillin on viability and levels of intracellular ATP were studied in bacterial cultures and close relationship between intracellular ATP levels and viability was demonstrated. The connection between the effects observed and MIC values is discussed. The possibility of using the phenomenon for rapid antibiotic susceptibility testing was studied in clinical isolates incubated for 2 hours followed by luciferase assay of intracellular ATP A positive correlation was demonstrated between ampicillin-induced decreases in intracellular ATP and inhibitory zone diameters, as measured by the agar diffusion technique.

Key words Adenosine triphosphate; intracellular levels bacterial cultures ampicillin.

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Received 17.xi.76 Accepted 7.xii.76

The simplicity and high sensitivity of the firefly luciferase ATP assay and the fact that it can easily be automated has made it an attractive alternative to classical microbiological methods (1 2, 6)

Since ATP is a central metabolite in living cells, the level of intracellular ATP is strictly controlled, and variations in cellular ATP content rarely exceed one order of magnitude (6) The determination of intracellular ATP can afford an estimation of the number of cells for instance in a bacterial culture. In principle, it should be possible to use assay of intracellular ATP to assess antibiotic induced inhibition of growth as an alternative to, for example turbidity measurements.

With the aim of investigating the possibility of using ATP assays for measuring antibiotic effects on microbial cells, the effect of ampicillin on ATP levels in bacterial cultures was studied. Intracellular and extracellular ATP levels were correlated with inhibition of growth and concentration of antibiotic.

Finally the possibility of using the method as a rapid antibiotic susceptibility test was investigated and compared with an agar diffusion technique in a material consisting of bacterial strains isolated from clinical specimens.

MATERIAL AND METHODS

Analytical Equipment

The luminometer used in the luciferase assay of ATP has been described previously (4)

Registration of maximum light intensity was made by a digital peak holding memory voltmeter model VID (G Gay Milan, Italy)

Analytical Reagents

Firefly luciferase (FLE 50) apyrase, grade *II* (crude) and adenosine-5 triphosphate were purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A

Ampicillin (ampicillin sodium, Doctacillin®) was purchased from Astra, Södertälje Sweden.

Other reagents were of analytical grade

Luciferase reagent was prepared as described previously (4) and could be used for 2–3 days if kept refrigerated. Apyrase stock solutions were prepared daily consisting of 0.2 per cent apyrase and *0 2 per cent triton x 100 made up in nutrient broth* (Difco) containing 4 mM $CaSO_4$

Microorganisms

Sarcina lutea (ATCC 9341) *Staphylococcus albus* (KS 462) and *Proteus mirabilis* (LU 5) were incubated in nutrient broth on a rotatory shaker at 37 C for 16–18 h, by which time the cultures were essentially stationary For the experiments shown in Fig 6 isolates from clinical specimens were selected to cover a wide range of ampicillin susceptibility

Sensitivity Testing

Sensitivity testing was performed using the broth *dilution and agar diffusion techniques (3) as indi*cated in the figures

Ampicillin Treatment of Bacterial Cultures

16–18 h stock cultures were diluted with fresh nutrient broth to contain the number of bacteria indicated in the figures. The diluted cultures were incubated at 37 C on a rotatory shaker in the presence of various concentrations of ampicillin. In the experiments depicted in Figs. 2–3 culture volumes of 50 ml in 250 ml Erlenmeyer flasks were used In Figs. 4–6 2.5 was incubated in 4 ml polystyrene tubes Aliquots were removed at hourly intervals for determination of the numbers of bacteria and intracellular and/or extracellular ATP concentrations.

Determination of Number of Bacteria

The numbers of bacteria were determined as colony forming units (CFU) by plating on nutrient

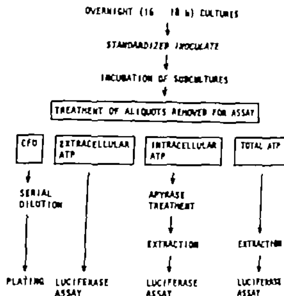


Fig 1 Schematic diagram of assay procedures. Experimental details given in text.

*agar (Difco) 0 1 ml of a serial dilution in physio*logical saline from 0 1 ml samples. Duplicate plates from the 10^{-3} and 10^{-4} dilutions were incubated overnight at 37 C and counted.

Luciferase Assay of ATP

Assays were performed on 1 ml samples consisting either of untreated bacterial culture or H_2SO_4 extracts. The samples were pipetted into 4 ml polystyrene tubes and placed in the dark chamber *of the luminometer* Luciferase reagent (0 4 ml) was injected manually into the sample by means of a 1 ml syringe and the maximum light intensity was recorded. *Standards with known amounts of* ATP and reagent blanks were assayed in each series All assays were performed in duplicate.

Determination of Extracellular Plus Intracellular ATP

1 ml aliquots from the bacterial cultures were extracted with 1 ml 0.3 M H_2SO containing 2 mM EDTA as described previously (5) The extract was neutralized before assay by a fivefold dilution in 6.5 mM KOH 20 mM tris base 2 mM EDTA followed by a further fi e-fold dilution in 20 mM tris/SO_4 pH 7 75 containing 2 mM EDTA.

Determination of Intracellular ATP

1 ml aliquots from the bacterial cultures were incubated for 10 min at 37 C with 1 ml of the apyrase stock solution. In control experiments, *this treatment with the ATP hydrolyzing enzyme*

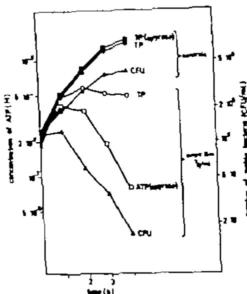


Fig 2 Effects of ampicillin on ATP levels and cell viability (CFU) in cultures of *S lutea.* CFU (Δ, ▲) determined at hourly intervals in control (filled symbols) and a culture exposed to 10 g/ml ampicillin (open symbols) Aliquots extracted with H_2SO following apyrase treatment (□, ■) or without apyrase treatment (○, ●) were assayed for ATP

apyrase resulted in complete elimination of added ATP The treatment had essentially no effect on control cultures but resulted in the disappearance of some ATP from ampicillin-treated cultures. Preliminary studies, in which triton 100 was included, resulted in more reproducible results, presumably due to lysis of cells with ampicillin-induced cell wall defects. After apyrase treatment, 1 ml aliquots were extracted with H_2SO_4, as described

Determination of Extracellular ATP

1 ml aliquots from the bacterial cultures were placed directly into the luminometer and the luciferase assay was performed without preceding extraction of ATP This simplified assay was shown to result in accurate determinations of extracellular ATP verified by assay of culture filtrates and supernatants from centrifuged samples. Thus, only extracellular ATP reacts with the luciferase, which presumably does not enter the bacterial cells.

Calculation of Assay Results

Sample ATP levels were calculated by using assays of standard amounts of ATP as reference and correcting for background luminescence.

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Interference with the assay resulting from bacterial debris and/or buffer salts present in the extracts, was corrected for by using an internal standard technique (4)

The entire assay procedure is outlined schematic in Fig. 1

RESULTS

Growth rates (CFU) and ATP levels in cultures of *S lutea* were determined at hourly intervals.

The results depicted in Fig. 2 show that, in a control culture growth is paralleled by an increase in ATP levels In a culture exposed to a strongly inhibitory concentration of ampicillin, the ATP level increases for two hours, after which the level is constant. Since

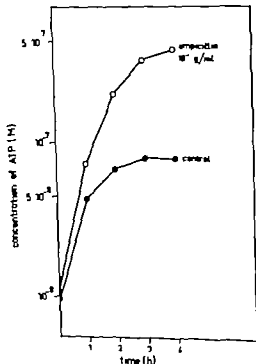


Fig 3 Accumulation of extracellular ATP in cultures of *S lutea.* Incubation mixture contained 1.0×10^6 CFU/ml at the beginning of the experiment. A control (●) and a culture exposed to 10^{-7} g/ml ampicillin (○) were assayed for the presence of ATP without preceding apyrase treatment or extraction with H_2SO

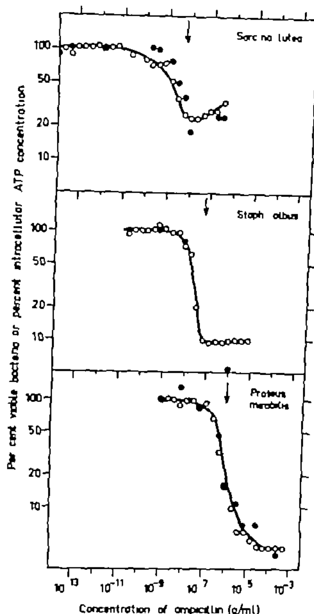


Fig 4 Dose/response relationship of the effect of ampicillin on cell viability (CFU) and intracellular ATP levels, in cultures of *S lutea* *S albus* and *P mirabilis* related to MIC values CFU (●) and ATP (○) values are related to control values (100 per cent) obtained in the absence of ampicillin. Arrows indicate MIC values obtained by the broth dilution technique

the number of viable cells gradually decreases during incubation, ATP levels cannot be correlated with CFU under these experimental conditions.

If the ampicillin-exposed culture of *S lutea* is subjected to treatment with apyrase part of the ATP is eliminated. The remaining ATP is now parallel with the decrease in CFU The observed effect of apyrase indicates the existence of extracellular ATP Consequently cultures of *S lutea* exposed to 10^{-7} g/ml ampicillin were assayed for the presence of extracellular ATP Fig 3 shows that extracellular ATP is accumulated in the culture and that the presence of ampicillin results in strongly increased levels of extracellular ATP The amount of extracellular ATP found in the ampicillin-treated culture corresponds closely to the amount of ATP lost after apyrase treatment (cf Fig 2)

In a series of experiments, cultures of *S lutea* *S albus* and *P mirabilis* at a density of approximately 3×10^7 CFU/ml, were incubated for two hours with serial dilutions of ampicillin. CFU were determined, and after incubation with apyrase and extraction with H_2SO_4 intracellular ATP was assayed. Fig 4 shows the close correlation between the decrease in CFU and the decrease in intracellular ATP levels with increasing concentrations of ampicillin MIC values correspond to ampicillin concentrations with pronounced effect on intracellular ATP

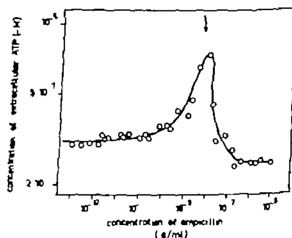


Fig 5 Dose/response relationship of the effect of ampicillin on accumulation of extracellular ATP in cultures of *S lutea* ATP was determined after 2 h incubation without preceding apyrase treatment or extraction with H_2SO_4 Bacterial content at the beginning of the experiment 1.0×10 CFU/ml Extracellular ATP level in a control after 2 h incubation 3.1×10^{-7} M Arrow indicates MIC value obtained by the broth dilution technique

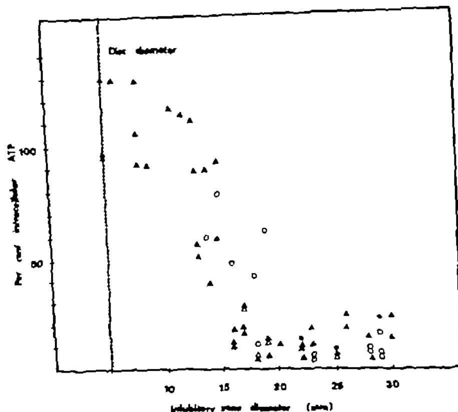


Fig. 6. Relationship between ampicillin effects on intracellular ATP levels and in vitro susceptibility testing. Clinical isolates of 33 strains of Coliform bacteria (Δ) 15 Staphylococcus sp. (○) 10 Enterococcus sp. (●) and 10 Proteus sp. (▲) were investigated.

The dose/response relationship of ampicillin concentration and extracellular ATP levels in cultures of *S. lutea* was examined in the experiment depicted in Fig. 5. The accumulation of extracellular ATP takes place at ampicillin concentrations approximately corresponding to those resulting in decreasing levels of intracellular ATP as shown in Fig. 4.

The relationship between effects of ampicillin on intracellular ATP and ampicillin susceptibility in a material of 68 clinically isolated bacteria is shown in Fig. 6.

Nutrient broth containing 10 μg ampicillin/ml was inoculated with overnight cultures of the clinical isolates to a final density of approximately 1×10^6 CFU/ml. Controls without ampicillin were inoculated simultaneously. Following incubation for 2 hours at 37 C, intracellular ATP was determined in all cultures and plotted as percentage of control values. Inhibitory zone diameters were determined by agar diffusion technique (3) with discs containing 10 μg ampicillin.

Division of the material into groups of low intermediate, and strong effects on intracellular ATP levels suggests a relation to ampicillin susceptibility expressed as inhibitory zone diameters.

DISCUSSION

The results of the present study indicate that determination of intracellular ATP may afford an early indication of disturbed cellular metabolism. A parallelity exists between the occurrence of extracellular ATP and effects on intracellular ATP levels and cell viability

The effects on intracellular ATP are observed at ampicillin concentrations below MIC values of the bacterial strains tested, and are possible to register within approximately two decades of ampicillin concentration

In S lutea the effect of ampicillin on intracellular ATP levels was shown to reflect a leakage of ATP presumably as the result of membrane damage. However in many bacterial strains accumulation of extracellular ATP may be prevented by the presence of ATP-ase activity

Luciferase assay of ATP is technically simple and the rapidly observable effects of ampicillin on intracellular ATP levels fulfill the technical requirements for a rapid susceptibility testing method The close relationship of antibiotic effects on viability and intracellular ATP levels, and its connection to MIC values as demonstrated in the model experiments, indicates a possibility of designing a susceptibility testing system based on luciferase assay of intracellular ATP

The results obtained in a clinical material (shown in Fig 6) indicate that it might be practicable to utilize this approach for rapid grouping of organisms according to antibiotic susceptibility Further work will be directed towards investigation of the clinical relevance of susceptibility groups based on assay of intracellular ATP

The technique might also be useful for determination of antibiotic levels in clinical specimens, utilizing the linear part of curves such as those shown in Fig 4 Studies aimed at exploring the practicability of these applications have been initiated and observations have been extended to include several bacterial strains and antibiotics with different modes of action.

This work was supported by the *Swedish Board for Technical Development*

The skilful technical assistance of Miss *Maud Karlsson* and Mrs. *Ann-Charlotte Ericson* is gratefully acknowledged.

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LACK OF MEASURABLE COMPLEMENT FIXING ANTIBODIES AGAINST VIRAL ANTIGENS

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Riski, H., Pyrhönen, S., Wager, O. & Penttinen, K. Lack of measurable complement fixing antibodies against viral antigens. Acta path. microbiol. scand. Sect. B, 85: 167–173, 1977.

A serological and clinical study was performed to find the common features of 130 patients without antibodies against 11 or more different antigens in the complement fixation (CF) test. These patients (= 1.6 per cent) were discovered during the screening of 8,021 adult patients. Rheumatoid factor(s) (RF) were found in the sera of 113 of the patients. In the remaining 17 patients no common serological or clinical markers were found. Myeloma M components were found in three cases. The lack of measurable CF antibodies in RF positive cases was apparently due to the inhibitory effect of RF( ) in the CF test. This was indicated by a positive reaction in CF after centrifugal separation of IgM and IgG fractions and also by the detection of antibodies using immunodiffusion method. Possible immune complexes were sought using the platelet aggregation test, which was positive for the sera of 47 (= 37 per cent) of the patients. The clinical diagnosis of the 130 CF-nonreactors was rheumatoid arthritis (RA) (ARA criteria) in 23 cases and pulmonary diseases in 65 cases. In a comparison group of equal size there were only 3 RA patients and 15 with pulmonary disease. RA was thus found in 20 per cent of the RF positive CF-nonreacting patients. In the comparison group of 52 RA patients 8 CF-nonreactors were found (= 15 per cent). This suggests that the effect of RF( ) from RA patients in CF reaction varies greatly.

Key words: Complement fixing antibodies; rheumatoid factor; viral antibodies; Fc part.

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Received 1.iii.76 Accepted 1.iii.76

One of the commonest methods used in the diagnosis of virus infections is the complement fixation (CF) test. A complete lack of the antibodies measured by the CF test is improbable: antibodies against herpes, cytomegalo, influenza, adeno or mumps viruses are found in a large proportion of the adult population. Statistically CF-nonreactive sera are an exceptional group. The nonreactivity of serum in the CF test is of significance in the reliability of diagnostic virus serology. It may also be a sign of host responses which have possible immunopathogenic importance.

To obtain more information about the nature of the CF-nonreactors 130 successive

cases were analyzed using various tests for rheumatoid factor and the platelet aggregation (PLA.) test which has been used to detect immune complexes (IC) (4)

TABLE 1 *Age Distribution of the 130 CF Nonreactors*

Age (years)	Number of cases Male	Female	Total
20–29	8	9	17
30–39	12	5	17
40–49	13	5	18
50–59	14	14	28
60–69	13	16	29
70–	10	11	21
Total	70	60	130

MATERIAL AND METHODS

Material

In 1972 paired serum samples from 4 735 subjects, and in 1973 from 6640 were sent from Finnish hospitals and laboratories to the Department of Virology University of Helsinki, to be screened for viral antibodies. 8,021 paires were from adults (over 20 years old) and these sera were studied further The age distribution of the 130 CF-nonreactors found is shown in Table 1 When the age distribution of the 130 CF nonreactors is compared with that of the 7,891 CF-reactors, there was no significant difference in age distribution between the two male groups in the female groups, however CF nonreactors were clustered among the over fifties (Table 2)

The diagnoses for the patients were obtained from hospital records. When a patient had been given several clinical diagnoses, he was placed in one of the following main groups rheumatoid arthritis, other "connective tissue" diseases, hypo-

TABLE 2 *Percentual Age Distribution of the Subjects over 20 Years whose Sera Were Sent for CF screening Test and the Age Distribution of CF-nonreactors*

		No of subjects	Percentage of total in each age group 20–29 (years)	30–39	40–49	50–59	60–69	70-
Female	All subjects (CF nonreactors excluded)	4314	26	19	14	15	13	13
	CF nonreactors	60	15	8	8	23	27	18
Male	All subjects (CF non-reactors excluded)	3577	16	16	20	18	20	10
	CF nonreactors	70	10	17	19	20	19	14

TABLE 3 *RF Serology of CF-nonreactors and Control Groups*

RF tests	Titer	All CF nonreactors (130) No. posit / No tested	%	CF nonreactors with Rheumatoid arthritis (23) No posit./ No. tested	%	Total CF nonreactors RA excluded (103) % posit.	Classical Rheumatoid arthritis (52) (Control group) % posit	Blood donors (117) (Control group) % posit
Latex	≥ 8	(106/130)	82	(21/23)	91	0	92	5
	≥ 32	(80/130)	62	(19/23)	83	57	82	0 8
	≥ 128	(7/130)	5	(5/23)	21	2	63	0
Waaler	≥ 16	(55/125)	44	(18/21)	78	36	73	0
Rose	≥ 512	(16/129)	12	(8/23)	35	7	46	0
Ripley	≥ 16	(92/120)	77	(19/20)	95	73	85	25
	≥ 512	(65/120)	54	(19/20)	83	46	65	0 9
Baboon	≥ 16	(92/120)	77	(18/19)	95	3	91	6
	≥ 512	(46/120)	38	(17/19)	90	29	74	0

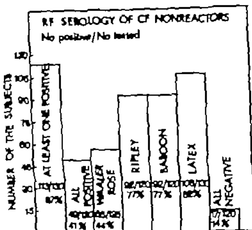


Fig 1 RF Serology of CF-nonreactors

gammaglobulinaemia or malignant neoplasms. If none of these diagnoses had been given, the patients were grouped according to the acute infection diagnosed. Patients with chronic infections were grouped according to the underlying disease when known.

To obtain a comparison group we paired our nonreactors with age and sex matched subjects, whose paired sera had been sent for viral antibody screening at the same time.

Screening Test for Complement Fixing Antibodies

The following 11 viral antigens were used in the screening test: herpes simplex type 1 (grown in BSC-1 cells), varicella, cytomegalo viruses (both grown in primary human embryonic fibroblasts), adeno type 5 (Bristol-HeLa cells), influenza A (and B) (chick embryos, Commercial products, Orion Diagnostica, Finland), parainfluenza 1 (chick embryos, Orion), mumps (chick embryos, Orion), respiratory syncytial viruses (U-cells), measles (Vero-cells, Orion), poliovirus type 2 (U-cells), ornithosis (commercial "TRIC CF A-11 Statens Seruminstitut, Copenhagen, Denmark). Also lipid mycoplasma antigen (Orion) was included.

A microtechnique with disposable U-microplates (Meda plates by Dansel & Co Helsinki, Finland) was used. All sera were diluted in veronal buffer (VB) diluent (8.5 g of NaCl, 0.375 g of Na barbital, 0.575 g of diethylbarbituric acid, 0.168 g of $MgCl_2 \times 6H_2O$ and 0.0038 g $CaCl_2$ per liter). The haemolytic system consisted of 2 per cent sheep erythrocytes and 2 full units of haemolysin. Two full units of complement, specifically titrated against each antigen, and four units of antigen were used. The initial dilution of serum was 1/8, and the reagents were pipetted at room temperature. 0.025 ml of diluted serum and antigen were incubated for 45 min at 4 C and complement was then added. The haemolytic system was added after overnight incubation at 4 C. The plates were further incubated for 1 h at 37 C and read by visual estimation of the degree of haemolysis.

Test for Rheumatoid Factor(s) (RF)

Human ORh positive cells were coated with rabbit antihuman erythrocyte serum (Waaler Rose), with baboon antihuman fibroblast serum ("baboon") or with human anti-D (Ripley). The latex titration was carried out with Hyland's RA test latex-globulin reagent. For all titrations U-microtiter equipment was used. Additional details are described elsewhere (7).

Platelet Aggregation Test (PLA)

The separation of platelets was made by differential centrifugation as described previously (6). The platelets were used on the day of preparation, 0.025 ml of the serum dilution and 0.025 ml of used buffer being pipetted on to a microplate well. 0.05 ml of platelet suspension was then added. The platelets were allowed to stand at 5-10° C for 18-20 hours and read against dark background illumination (4). Controls for the sensitivity of different individual platelet lots in all the series were titrations against NIP-BSA antigen and anti-NIP antibody complex and against heat-aggregated human IgG preparation.

Immunodiffusion Test (ID)

Immunodiffusion test was carried out using the micromodification of the gel double diffusion previously described (8).

RESULTS

I RF Activity of CF nonreactors

Of the 130 CF-nonreactors detected, 113 (= 87 per cent) gave a positive result in at least one of the RF tests (Fig. 1). Of these tests the latex test was most often positive (82 per cent). The corresponding percentage in an unselected series of 100 sera sent for viral antibody screening was only 7 per cent. The Waaler Rose positivity in the CF-nonreactor group was 44 per cent. The titers of both tests were lower in the CF-nonreactor group than in two RA groups (Table 3).

TABLE 4 *Direct Platelet Aggregation Test*

	No positive/ No. tested	%
CF nonreactors	47/130	36
RF positive CF-nonreactors	40/113	35
RF negative CF nonreactors	7/17	41
CF nonreactors with rheumatoid arthritis	12/22	53
Classical rheumatoid arthritis (comparison)	10/43	?3
Blood donors (comparison)	1/127	<1

II CF nonreactive Rheumatoid Arthritis Patients

23 (= 18 per cent) of the 130 CF non reactors fulfilled the ARA criteria for definite or classical rheumatoid arthritis (RA) (9) Five patients had classical RA the others definite RA The CF nonreactors with RA showed positive RF reactions in all RF tests more frequently than did the CF nonreactors without RA (Table 3)

III RF Activity in Comparison Groups

We compared the above results with those of 52 patients from the Rheumatoid Foundation Hospital Heinola, who fulfilled the ARA criteria for classical rheumatoid arthritis (RA) (RF serology is not included among these criteria) There were 3 RF negative and 8 CF nonreactors (= 15 per cent) in this group The CF nonreactors were all latex and Waaler Rose positive Classical RA patients showed positivity in the RF tests significantly more often than did CF nonreactors (including RA patients) This difference was evident at all titer levels of the Waaler Rose test and at high titers of the latex and baboon tests.

The second comparison group consisted of 117 blood donors whose RF tests gave fairly negative results compared to the other groups studied as shown in Table 3

IV Platelet Aggregation Test (PLA)

Because a large proportion of the CF non reactive sera contained measurable RF the presence of IgG immune complexes in the sera was also to be expected (13) As IgG complexes have been shown to cause aggregation of human platelets (6) the sera were tested for platelet reactivity A higher than normal incidence of direct PLA activity (Table 4) was found in all the various groups tested, irrespective of the presence or absence of detectable RF activity

V Determination of Antibodies by the Immunodiffusion (ID) Technique

Antibodies against adeno or influenza virus were studied from 41 CF nonreactive sera with the immunodiffusion (ID) technique Each of these sera had antibodies against one or the both of these viruses. Four sera with high antibody titers against adenovirus antigen in immunodiffusion were centrifuged on sucrose gradients. After separation of the IgM from IgG antibodies, adenovirus antibodies could be shown in all the sera also with the CF technique

VI Clinical Diagnosis

Our CF nonreactor group included 23 RA patients (8 in the comparison group) 5 patients had hypogammaglobulinaemia (as against two in the comparison group) and two of them had measurable RF The number of various pulmonary diseases was high 65 out of 130 patients had some kind of pulmonary disease compared to 15 in the comparison group. Table 5 shows only 43 in the pulmonary disease group because 12 patients also had RA and remaining 10 had some other main group" diagnoses.

Among the CF nonreactors, but without RF activity there were also 3 *patients with* M-components two IgA and one IgG One had IgA myeloma, but no myelomas have been found in the other two so far despite intense hospital examination. The three patients were RF negative and had a positive PLA. *reaction*. Two others without RF in their sera had hypogammaglobulinaemia, but for the remaining 12 subjects no specific explanation could be found for their CF non reactivity

TABLE 5 *Clinical Diagnosis*

		Material		Comparison group	
		Male	Female	Male	Female
Rheumatoid arthritis		10	13	1	2
Connective tissue diseases		3	2		1
Malignant neoplasms		5	1	6	2
Hypogammaglobulinaemia		1	4	1	1
Pulmonary diseases	Total no	43		12	
	Pneumonia	11	9	4	1
	Acute respiratory infection, acute bronchitis	1	4	2	
	Tuberculosis	7	1	2	
	Pleurisy	3	2		
	Other pulmonary diseases	2	3	1	2
Acute infections		13	12	15	17
Chronic infections or period after the infection proper		3	1	4	2
Other diseases		11	8	34	32
Total		70	60	70	60

DISCUSSION

We report a study of 150 adult patients among whom no viral antibodies could be detected in CF screening tests with 11 or more viral antigens. Such sera were found in about 1.6 per cent of all adult sera sent to our laboratory. This material partially overlaps with the 41 patients studied earlier (5), but the percentage of CF-nonreactors is slightly higher. Many of the viral diseases screened for the CF test are known to be very common, and CF antibodies against these viral antigens can be found in a large proportion of adults. In fact, if 80 per cent of adults have antibodies measurable in CF against the antigens of measles, mumps, influenza, herpes, adeno and cytomegalo viruses, the probability of someone lacking all these antibodies is about 1 in 15,000. Of course this non-reactivity in the CF test is a relative phenomenon. It clearly depends on the initial dilution of serum used in the laboratory and the borderline between reactive and nonreactive sera is not sharp. For instance the lowest dilution of serum may be positive for one antigen.

Low viral antibody titers in the CF test have been previously reported for rheumatoid patients. Detectable antibody titers have been lower in rheumatoid patients than in the control subjects tested for herpes simplex, measles and mumps antigens (12). *Smiley & Casey* (11) have studied herpes simplex, adeno, rubella, measles and psittacosis antibodies, but only the herpes simplex antibodies were lower in rheumatoid arthritis patients. Mean measles titers were 3 to 4 times higher in RA patients without rheumatoid factor than in RA patients with RF (3).

A large proportion (87 per cent) of the CF-nonreactive sera had measurable RF(s) in the present study, whereas only 7 per cent of the random sera sent for the screening test were RF(s) positive in the latex test. This implies that RF(s) may mask or decrease the "true" antibody titers in the CF test. RF is known to react with the Fc part of the IgG molecule and may thus block the closely situated complement site. In addition the following findings suggest that a common mechanism for the apparent absence of CF antibodies is inhibition of the CF reaction by RF(s). After separation of IgG from IgM RF(s) by sucrose gradient centrifugation

CF viral antibodies were found in the IgG fractions. Absorption of RF(s) with heat aggregated IgG converted CF nonreactive sera into sera which reacted with some of the antigens tested (5)

In the series investigated there were 17 sera which did not have measurable amounts of RF(s) Explanations other than RF(s) must therefore be considered as the reason for CF nonreactivity in these cases. Among these 17 patients, there were two patients with hypogammaglobulinaemia and three with M-components, which may explain the CF nonreactivity of these sera The remaining 12 may by coincidence lack these antibodies, or factors other than RF(s) may interfere with the CF reaction.

It is known that one prerequisite for the production of RF(s) is appearance of IgG immune complexes. When these sera were tested using the PLA test, which is known to measure ICs, a positive reaction was frequently found (i.e. in 37 per cent of the sera) We do not know how frequently the PLA reaction was due to ICs proper and in which sera there were other platelet stimulating factors such as aggregated IgG antiplatelet antibodies etc. From sucrose gradient studies, it is evident that at least some of these PLA reactions were due to ICs.

The Fc part of the IgG antibody is known to induce platelet aggregation as well as to contain the binding site for RF and CF Some purified RF(s) can effectively inhibit the IgG immune complex mediated PLA reaction and also fixation of C by IgG complexes. Consequently it could be expected that sera containing RF(s) would not induce PLA reaction and could inhibit CF The presence of both RF(s) as well PLA activity and CF reactivity concomitantly in a serum could be explained by assuming that the patient's own RF(s) are ineffective in some interactions with the Fc part This may be due to varied specificities, affinities or relative amounts of the reactants. Because of our selection the present series contains sera with RF-specificity against that domain of the Fc part in which a complement fixation site is located

The practical implications of the present study are that the RF(s) in sera must be considered seriously when studying CF viral antibodies. This applies to both routine diagnostic laboratory work and seroepidemiological studies with RF(s) containing sera. The series presented shows at least some cases of infectious diseases, such as the five cases of serous meningitis, in which the aetiological diagnosis might be masked by RF(s) To avoid misleading titers, the RF(s) should be removed prior to testing by absorption of RF(s) from serum with aggregated human γ-globulin (12) or with kaolin (2) or disruption of IgM RF(s) with 2 mercaptoethanol. Comparative studies using different methods should also be performed.

From the clinical point of view it is interesting to note that about half the patients in the present series had pulmonary diseases. This incidence was fourfold higher than in the comparison patients. Previously it has been shown that in some pulmonary diseases e.g chronic bronchitis, tuberculosis and alveolitis, RF(s) are frequently found (1) Abnormal pleuropulmonary changes have been shown by x ray examination to be twice as frequent among rheumatoid patients with high titers of RF(s) as among those RA patients without RF(s) (10) These findings may also suggest that RF(s) have some immunopathogenic significance.

Further evaluation of the function of RF(s) in various infectious and pulmonary diseases will be revealing Nonexisting or very low titers of CF antibodies are indicative of an immune complex mediated systemic disease the development of which may be difficult to distinguish from acute infections.

This work was supported by grants from the *Sigrid Jusélius Foundation* and the *Finnish Antituberculosis Association*. We gratefully acknowledge the receipt of RA sera from the *Rheumatoid Foundation Hospital* Technical assistance of Mrs. *Anja Kallionpää* and *Ann Maj Svensson* is gratefully acknowledged

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Acta path. microbiol. scand Sect B 85 174–176, 1977

BRIEF REPORT

THE TESTING OF THE ANTIBIOTIC SENSITIVITY OF BACTERIA ON AN AGAR MEDIUM THE PROBLEM OF A DOUBLE ZONE OF INHIBITION

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Korkeala, H & Pekkanen, T J The testing of the antibiotic sensitivity of bacteria on an agar medium The problem of a double zone of inhibition. Acta path. microbiol scand. Sect. B 85 174–176 1977

When the sensitivity of *Micrococcus luteus* ATTC 9341 to streptomycin, erythromycin, oleandomycin and spiramycin was tested by an agar diffusion method using antibiotic impregnated filter paper disks on unbuffered Penassay Seed Agar two zones of inhibition were observed around the disks after an incubation period of 24 hours at 30 C. The pH of the *M luteus* seeded Penassay Seed Agar was measured before and after 24 hours incubation at 30 C and found to be 6 6 and 8 7 respectively When the Penassay Seed Agar was buffered to pH 6 1 and the sensitivity of the microbe to the antibiotics was tested as before no double zones of inhibition could be observed. The phenomenon of the double zones of inhibition may possibly be due to the pH increase of the medium from a relatively low level to the optimum range of activities of the antibiotics during the incubation period

Key words Sensitivity testing antibiotics effect of pH

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Received 1 vii 6 Accepted 22 xii.76

The sensitivity of *Micrococcus luteus* ATCC 9341 to streptomycin erythromycin oleandomycin and spiramycin was tested by an agar diffusion method ( ) using antibiotic impregnated filter paper disks (Schleicher & Schull Dassel GFR, Ø 12 7 mm) on Penassay Seed Agar (Difco Laboratories, Detroit Michigan USA) The potency of the disks was as follow streptomycin 40 µg erythromycin 20 µg oleandomycin 50 µg and spiramycin 50 µg per disk The size of the inoculum was the same throughout the experiments, 0 2 ml of a bacterial suspension containing about 3 × 10⁸ colony forming units per ml The minimum inhibitory concentration (M I C) of the antibiotics against the microbe was determined by a plate dilution test (1) The results are presented in Table 1 The microbe was also tested for inducible erythromycin resistance (3) and found to be not resistant.

After the full incubation period (24 hours at 30 C) two zones of inhibition were always observed around the antibiotic-impregnated filter-paper disks The zone immediately around the sample was clear without visible growth of bacteria The second peripheral zone surrounding the clear zone showed bacterial growth but could be clearly distinguished from both the surroundings and the inner zone (Fig 1) The two zones were most clearly seen around the streptomycin disks. The phenomenon was not observed when the sensitivity of the microbe to penicillin and tetracyclines was similarly tested, using disks containing 0 1 IU penicillin and 40 µg tetracyclin respectively

The pH of the *M luteus* ATCC 9341 seeded Penassay Seed Agar was measured after 24 hours incubation at 30 C in a 50 ml decanter in which the agar from the petri disk was minced and diluted with a few drops of distilled water The pH

was found to be 8.7 Before incubation the pH was 6.6. The antimicrobial pH optimum of streptomycin is 7.5–8.0 and that of macrolide antibiotics over 8 (4). The corresponding pH optimum of penicillin is about 4–6 and of tetracyclines about 6.1–6.6 (4, 2).

We thought that the presence of the two zones of inhibition might be explained as follows. The clear inner zone is due to high antimicrobial activity during the initial phase of incubation. When the pH of the substrate increases during incubation, it approaches the optimal antimicrobial range of activity of streptomycin and the macrolide antibiotics, and the antibiotic further inhibits the growth of the microbe in the area of the peripheral zone, although the concentration of the antibiotic there is lower than in the central area.

TABLE 1 *The M.I.C.-values (µg/ml Substrate) of certain Antibiotics against* Micrococcus luteus *ATCC 9341 on Penassay Seed Agar Unbuffered and Buffered to pH 6.1*

	Penassay Seed Agar	
	Unbuffered	Buffered to pH 6.1
Streptomycin	3.20	12.80
Erythromycin	0.03	0.10
Oleandomycin	2.00	4.00
Spiramycin	0.03	0.10

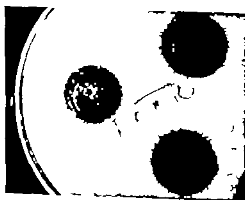


Fig. 1 Two inhibition zones of the growth of *Micrococcus luteus* ATCC 9341 on Penassay Seed Agar after 24 hours incubation at 30° C around filter-paper disks containing 40 µg of streptomycin. The diffusion time of the streptomycin into the substrate before incubation was 2 hours at 4° C.

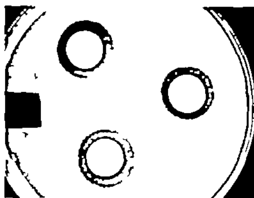


Fig. 2 Absence of two inhibition zones of the growth of *Micrococcus luteus* ATCC 9341 on Penassay Seed Agar buffered to pH 6.1 after 24 hours incubation at 30° C, around filter-paper disks containing 40 µg of streptomycin. The diffusion time was similar to that given in Fig. 1.

To test this hypothesis, the Penassay Seed Agar was buffered by phosphate buffer (2 g K_2HPO_4 and 8 g KH_2PO_4 to 1000 ml of the substrate) to pH 6.1. The *M. luteus* ATCC 9341 was tested for sensitivity to the antibiotics as before using the buffered substrate. No double zones of inhibition could be observed (Fig. 2). The final pH of the buffered *M. luteus* seeded substrate after 24 hours incubation at 30° C was about 6.5.

The M.I.C.-values of the antibiotics tested against the microbe were also tested on the buffered medium as before. The results (Table 1) show, as expected, that the M.I.C.-values for the antibiotics are greater on the medium buffered to pH 6.1 than on the unbuffered medium with an initial pH 6.6. The growth capacities of the test microbe on the buffered and unbuffered Penassay Seed Agars were studied by inoculating a similar volume of a suspension of the test microbe on ten buffered and ten unbuffered mediums. The logarithmic means of the number of colonies after 24 hours incubation at 30° C on the respective agar mediums did not differ significantly (Students t-test).

Thus it may be concluded that the double zones of inhibition of microbial growth which sometimes appear when microbes are tested for sensitivity to streptomycin or macrolide antibiotics by an agar diffusion method may possibly be due to the pH increase of the medium from a relatively low level to the optimum range of activity of the antibiotic during the incubation period. To the authors' knowledge no similar explanation for the phenomenon in question has been given in the literature.

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MICROBIAL AGGREGATE CONTAMINATION OF WATER LINES IN DENTAL EQUIPMENT AND ITS CONTROL

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Kelstrup, J., Funder Nielsen, T. D. & Theilade, J. Microbial aggregate contamination of water lines in dental equipment and its control. Acta path. microbiol. scand. Sect. B, 85: 177–183, 1977.

Water from some dental clinics has been examined and found to be discoloured, badly tasting and with a foul odour. Moreover, brown or black flakes were often present in tap water as well as in the water lines of dental equipment. Examination by phase-contrast and electron microscope showed the flakes to consist of aggregated fungi and bacteria, and similar structures were found in a layer on the inner surfaces of the clinics' water tubes and pipes. The ultrastructure of some aggregating microorganisms, including fungal hyphae and sheath-forming and stalked bacteria, was studied in detail, and several modes of aggregation were suggested. Cultivation of contaminated water samples revealed the presence of filamentous fungi, including *Cladosporium* and *C. phaeosperium*, and of non-fluorescent *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Moraxella* (?). Removal of microorganisms from the walls of the tubing was effectively accomplished by rinsing with the non-corrosive solution of 4 per cent Tween 80, coloured with Ponceau 4 R.

Key words: Contaminated water lines; fungi; bacteria; dental clinics.

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Received 10.xii.76 Accepted 10.xii.76

It is a phenomenon of profound oecological significance that certain microorganisms, including non-epiphytic microbes, attach to solid surfaces and build up masses generally consisting of different species of organisms (2). In some cases the attachment of microbes may cause obnoxious problems. An example is the occurrence in utility water of microbial products and flocks of microorganisms, derived from a layer of microbes on the interior walls of water pipes and tubes.

This communication describes the presence in dental equipment and tap water of plugs of aggregated microorganisms which could lead to a complete clogging of the water supply to the equipment used in dentists' operating rooms. The occurrence of microorganisms on the internal surface of water pipes and tubes was demonstrated and a characterization of microbes present within the aggregates was attempted. Selected microorganisms were identified, comprising both fungi and bacteria, and means of removing the obstructing microorganisms are proposed.

MATERIALS AND METHODS

Samples Material was obtained from the water supply system of the Royal Dental College, Aarhus and from dental clinics in that and other cities. The samples were obtained from the inner walls of polyvinyl chloride and polyethylene tubes of water requiring dental equipment, from the water of such tubes, and from cold water taps. Flakes of "dirt" from water were collected on filter paper and kept moist in plastic bags during transport. Water from the dental school was received and inspected at the laboratory less than an hour after sampling all other samples were usually inspected within a few hours and at any rate within 24 hours.

Light and electron microscopy Wet mounts of the samples from tubes and water were examined by phase-contrast microscopy For electron microscopy the samples were suspended in a few drops of distilled water and disintegrated with a platinum loop. With the same loop a portion of the suspension was transferred to carbon reinforced Formwar coated grids and allowed to dry The grids were then placed on drops of the solutions used for negative staining. Each drop was placed on dental wax. The grids were removed after 20–30 seconds and excess solution sucked away from the grids with filter paper after which the grids were air dried. The staining solutions were either 1 0 per cent ammonium molybdate, adjusted to pH 7 0 with NH_4OH, or 2 0 per cent phosphotungstic acid adjusted to pH 7 0 with KOH. The grids were examined in a Philips EM 301 electron microscope operated at 60 kV

Cultivation The samples were homogenized in 0 145 M NaCl in glass tissue homogenizers with teflon pestles and cultivated on agar plates. Growth media were Trypticase Soy Agar (Balt. Biol. Lab.) for bacteria and Sabauraud Dextrose Agar (Difco) with 20 i.u penicillin and 40 µg streptomycin per ml for fungi, such rather rich media being employed in order to resuscitate injured organisms (1) The plates were incubated in moist air at room temperature or at 30 C from one day to several weeks. Selected colonies were isolated and purified The fungi from these were identified at the Mycological Department, Statens Seruminstitut, Copenhagen and the bacteria at the Institute of Veterinary Microbiology and Hygiene at the Royal Veterinary and Agricultural University Copenhagen.

Additional procedures Removal of material adhering to plastic tubes of dental equipment was attempted by treating 1 cm long pieces of tubing on a whirlimixer for 30 s in 2 0 ml distilled water containing 1 0 per cent sodium desoxycholate, 0 04 M ethylenediamine-tetraacetate (EDTA) 20 per cent NaCl, 1 0 per cent H_2O_2, or Tween 80 in varying concentrations up to 4 0 per cent. The dye Ponceau 4 R was added to the solution used at a concentration of 40 µg/l in order to make the liquid easily visible The corrosive effect was tested by leaving test pieces of brass wire in the solutions for 18 hours before inspection under a dissecting microscope at 25 × magnification. Controls were kept in water or air Before being used in the corrosion test, the wire was cleaned by immersion in 66 per cent HNO_3 for 10 s, rinsed in water and finally dried.

RESULTS

The water samples obtained from dental equipment or from cold water taps at the clinics were often grey or brown and had a foul odour and bad taste. The inner walls of the water pipes and tubes examined were covered with a layer of black or brown material which appeared to consist of fungi and bacteria when examined in the phase-contrast microscope. Similarly the flakes present in the water contained these types of organisms. The flakes were black or brown and were difficult to disintegrate. Often they had dense centres so that details had to be studied at the periphery where fungal hyphae and spores were seen together with numerous bacteria some of which were motile (Fig 1)

Electron microscopy The results obtained with the two negative stains used did not differ significantly Numerous microorganisms of different types were seen in varying degrees of preservation (Fig 2 3) The microorganisms were often surrounded by a fibrillar network and had a morphology corresponding to either fungal hyphae or bacteria (Fig 2 3) The bacteria found were mostly short rods, but occasionally cocci, and in a few instances spirochetes were seen.

Some bacterial rods appeared in chains surrounded by sheaths. The sheaths sometimes showed a substructure of rings about 12 nm in diameter arranged in a hexagonal pattern (Fig 7) Other bacteria had long projections (stalks) at one end (Fig 3) and cross striations were evident in some of the stalks (Fig 6)

Between the microorganisms, material was found which contained long curved filaments with a diameter of 15–20 nm, resembling bacterial flagellae (Fig 5) Straight



Fig. 1. Phase-contrast micrograph from the periphery of flake obtained from tap water. Numerous bacteria intermingled with fungal hyphae (A) are seen in the centre of the illustration. Some bacteria (B) resemble sheath-forming bacteria. These bacteria are attached to fungal hyphae. Magnification × 960. The bar represents 20 μm.

thin filaments with a diameter about 3 nm were observed in some regions. These filaments showed a tendency to aggregate in bundles (Fig. 4).

Cultivation. Microorganisms derived from water samples obtained from eight dental units were examined. Eighteen isolates of fungi proved to belong to the genera *Cladosporium*, *Cephalosporium* or to be unspecified filamentous fungi. Eleven bacterial isolates were identified as *Pseudomonas* (non-fluorescent), *Aeromonas*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium* and tentatively *Moraxella*. Thus, with the exception of a single strain of *Moraxella (?)* all isolates were typical water or soil organisms, none of which are normally encountered in the human mouth.

Rinsing of tubes. Microscopy of the material obtained from the inner walls of tubings of dental equipment showed, as stated earlier, that it consisted of aggregated microorganisms. Rinsing of the tubes was consequently attempted with various compounds known to interfere with forces involved in microbial adhesion and aggregation (15). Only desoxycholate, H_2O_2, and Tween 80 were found to be effective, and Tween 80 in a high concentration (4 per cent) proved superior. This solution was found to be less corrosive than water, and 4 per cent Tween 80 with 40 μg Ponceau 4 R per litre (TPR) was therefore

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MATERIALS AND METHODS

Samples Material was obtained from the water supply system of the Royal Dental College Aarhus, and from dental clinics in that and other cities. The samples were obtained from the inner walls of polyvinyl chloride and polyethylene tubes of water requiring dental equipment from the water of such tubes, and from cold water taps. Flakes of "dirt from water were collected on filter paper and kept moist in plastic bags during transport. Water from the dental school was received and inspected at the laboratory less than an hour after sampling all other samples were usually inspected within a few hours and at any rate within 24 hours.

Light and electron microscopy Wet mounts of the samples from tubes and water were examined by phase-contrast microscopy For electron microscopy the samples were suspended in a few drops of distilled water and disintegrated with a platinum loop With the same loop a portion of the suspension was transferred to carbon reinforced Formwar coated grids and allowed to dry The grids were then placed on drops of the solutions used for negative staining Each drop was placed on dental wax. The grids were removed after 20–30 seconds and excess solution sucked away from the grids with filter paper after which the grids were air dried. The staining solutions were either 1 0 per cent ammonium molybdate, adjusted to pH 7 0 with NH_4OH, or 2 0 per cent phosphotungstic acid, adjusted to pH 7 0 with KOH. The grids were examined in a Philips EM 301 electron microscope operated at 60 kV

Cultivation The samples were homogenized in 0 145 M NaCl in glass tissue homogenizers with teflon pestles and cultivated on agar plates Growth media were Trypticase Soy Agar (Balt. Biol. Lab) for bacteria and Sabauraud Dextrose Agar (Difco) with 20 i.u. penicillin and 40 µg streptomycin per ml for fungi, such rather rich media being employed in order to resuscitate injured organisms (1) The plates were incubated in moist air at room temperature or at 30 C from one day to several weeks. Selected colonies were isolated and purified. The fungi from these were identified at the Mycological Department, Statens Seruminstitut, Copenhagen, and the bacteria at the Institute of Veterinary Microbiology and Hygiene at the Royal Veterinary and Agricultural University Copenhagen.

Additional procedures Removal of material adhering to plastic tubes of dental equipment was attempted by treating 1 cm long pieces of tubing on a whirlimixer for 30 s in 2 0 ml distilled water containing 1 0 per cent sodium desoxycholate 0 04 M ethylenediamine-tetraacetate (EDTA) 20 per cent NaCl 1 0 per cent H_2O_2, or Tween 80 in varying concentrations up to 4 0 per cent. The dye Ponceau 4 R was added to the solution used at a concentration of 40 µg/l in order to make the liquid easily visible The corrosive effect was tested by leaving test pieces of brass wire in the solutions for 18 hours before inspection under a dissecting microscope at 25 × magnification. Controls were kept in water or air Before being used in the corrosion test, the wire was cleaned by immersion in 66 per cent HNO_3 for 10 s, rinsed in water and finally dried.

RESULTS

The water samples obtained from dental equipment or from cold water taps at the clinics were often grey or brown and had a foul odour and bad taste. The inner walls of the water pipes and tubes examined were covered with a layer of black or brown material which appeared to consist of fungi and bacteria when examined in the phase-contrast microscope. Similarly the flakes present in the water contained these types of organisms. The flakes were black or brown and were difficult to disintegrate. Often they had dense centres so that details had to be studied at the periphery where fungal hyphae and spores were seen together with numerous bacteria some of which were motile (Fig 1)

Electron microscopy The results obtained with the two negative stains used did not differ significantly Numerous microorganisms of different types were seen in varying degrees of preservation (Fig 2 3) The microorganisms were often surrounded by a fibrillar network and had a morphology corresponding to either fungal hyphae or bacteria (Fig 2 3) The bacteria found were mostly short rods, but occasionally cocci, and in a few instances spirochetes were seen.

Some bacterial rods appeared in chains surrounded by sheaths. The sheaths sometimes showed a substructure of rings about 12 nm in diameter arranged in a hexagonal pattern (Fig 7) Other bacteria had long projections (stalks) at one end (Fig 3) and cross striations were evident in some of the stalks (Fig 6)

Between the microorganisms, material was found which contained long curved filaments with a diameter of 15–20 nm resembling bacterial flagellae (Fig 5) Straight



Fig 7 Part of a bacterial sheath which shows substructure of rings arranged in a hexagonal pattern. The cytoplasmic membrane (CM) of the bacterium within the sheath is seen at a distance from the lateral border of the sheath. Magnification × 100,000. The bar represents 0.1 µm.

tion of several microbial genera has been accomplished by exploring their adherence to glass. Several recent reviews have focused on the adhesion and aggregation of microorganisms (4 5 7 14) and the subject has been dealth with in detail by *Brock* (?)

The light and electron microscopical examination of the material contaminating the dental clinic water and water pipes and tubes revealed that it contained numerous microorganisms of various morphological forms. Fungi were prevalent in the aggregates, intermingled with bacteria, which were mostly short rods Some of them appeared in chains adhering to fungal hyphae, and examination in the electron microscope revealed that the rods were surrounded by sheaths, some of which displayed a characteristic ultrastructure Such orderly arrays of substructural units have been demonstrated in the cell wall or external sheaths of some microorganisms (8) Bacterial sheaths may be of significance in aggregating bacteria as they multiply (3)

Furthermore the examination demonstrated rodshaped bacteria which had cellular protrusions with characteristic cross striations, conforming to descriptions of stalked bacteria (12, 15 16) It is significant that such stalks may form sticky "holdfasts" with which they are able to adhere to solid surfaces.

The material between the microorganisms was seen to consist of a fibrillar substance. Curved fibrils with a diameter corresponding to that of bacterial flagellae suggest that part of the aggregates were formed by flagellated bacteria or disintegrated spirochetes, and that the shedded filaments contributed to the coherence of the aggregates. Other filaments were thin and straight and might represent fimbriae detached from organisms such as *Pseudomonas* and *Acinetobacter* which are known to possess fimbriae (9 10)

The abundance of microbial mats may have developed as a result of several cooperating conditions (i) The insufficient amounts of nutrients available in the environment, and (ii) the relatively low flow rate in some of the pipes and tubes of the water supply system of the buildings from which the samples were obtained. It has been observed that the minute amounts of organic matter in natural water show a tendency to be adsorbed on to a variety of inert solid surfaces, and that microorganisms in turn adhere to such sur



Fig 2–7 Electron micrographs of material similar to that in Fig 1 The material was negatively stained with ammonium molybdate or phosphotungstic acid.

Fig 2 A fungal hyphae is seen to the left. Rodshaped bacteria surrounded by sheaths are present at the top and to the right in the illustration. Protrusions (stalks) from other bacteria are seen in the centre of the field. Magnification × 7,000 The bar represents 1 µm.

Fig 3 Rodshaped bacteria, one of which has a projection (stalk) at one end. Magnification × 9,500 The bar represents 1 µm.

Fig 4 An aggregate of thin straight filaments each with a diameter of 3 nm Magnification × 72,600. The bar represents 0 1 µm.

Fig 5 Material including many long curved filaments with a diameter of 15–20 nm found between the microorganisms. Magnification × 51 500 The bar represents 0 1 µm.

Fig 6 Part of bacterial projection (stalk) which exhibits a characteristic cross striation. Magnification × 50 000 The bar represents 0 1 µm.

used routinely for rinsing the tubings of dental equipment.

Contaminated airotor tubes from more than a hundred dental units at the dental college were rinsed The tubes were filled with TPR, left for 2–3 hours, and subsequently washed with 40 volumes of water by connecting the tubes directly to the utility water hoses. This rinsing removed large amounts of black material that resembled microscopically the aggregates of fungi and bacteria previously found in the water

DISCUSSION

It is well known that microorganisms in nature tend to adhere to solid surfaces and, in turn, to each other In fact primary isola

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faces, where they use the organic material as nutrients (11, 17) Most likely, a secondary concentration of nutrients occurs on the already adherent microbes, thus leading to a continued adhesion of microorganisms (6)

In addition the water left stagnant in dental clinics during part of the day week ends or vacations provides excellent conditions for aggregation and for growth of microorganisms in the aggregates. Only when a sufficient flow of water is established is a loosely adherent slough washed off and may then leave the system (6) and may for example, clog up the narrow tubing of the airotor used by the dentist.

The isolation of microorganisms that are natural inhabitants of water soil or both, supports the content on that the utility water is the source of the contamination. Probably a layer of microorganisms is built up gradually on the inner walls of the water pipes and tubes, and the microorganisms of this layer could be responsible for the foul odour bad taste and the microbial flocks

Fortunately the problem can be overcome by simple measures. Effective rinsing can be accomplished by treatment with 4 per cent Tween 80 solution in water The possibility of dispersing aggregated microorganisms by non ionic detergents has been reported previously (5) In our hands, the Tween solution with Ponceau 4 R added (TPR solution) was found to be non-corrosive Furthermore this solution is inexpensive to prepare and can therefore be recommended for the removal of this kind of bacterial contamination from the water tubes of dental equipment and clinics. Naturally it would be a still better measure if the microorganisms could be prevented from gaining access to environments that are favourable for their adhesion and aggregation This could be achieved by mounting appropriate filters in the water pipes of buildings where such problems are likely to develop

Fungal and bacterial isolates were kindly characterized by Dr *J Bodenkoff* Mycological Department, Statens Seruminstitut, Copenhagen, and Dr *H Errebo Larsen* Institute of Veterinary Microbiology and Hygiene, Royal Veterinary and Agricultural University Copenhagen. The phase-contrast photomicrograph was provided by Dr *H P Philipsen* Department of Oral Pathology Royal Dental College Aarhus. We are grateful to Dr *A. Birch Andersen* Department of Biophysics, Statens Seruminstitut, Copenhagen for valuable suggestions and criticism during preparation of the manuscript. The study was supported in part by grants from the *Danish State Medical Research Council*

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(Merck) in distilled water was applied onto the agar surface of each plate, and reduction of tellurite was allowed to take place for 3 to 9 hours of additional aerobic incubation at 37 C. During this period the colonies were inspected regularly under a low power microscope to estimate the extent of reduction of the substrate. Strains showing a positive reaction were examined further by electron microscopy For this purpose, tellurite reduction was terminated by washing the colonies on the plates with tris-maleate buffer containing 0 01 M $CaCl_2$, pH 6.1 (VA buffer) (13) The colonies were subsequently prefixed in situ with 0 5 per cent glutaraldehyde in VA buffer for one hour at room temperature and then covered with one or two drops of warm (45 C) 1 per cent Noble agar (Difco) in VA buffer After solidification, small agar blocks, each containing single colony were cut out and fixed in 3 per cent glutaraldehyde in VA buffer for one hour at room temperature Further fixation in 1 per cent OsO_4 solution containing yeast extract-sodium acetate-peptone medium (5) followed by treatment with 2 per cent uranyl acetate (13) took place as described elsewhere (2) Dehydration was carried out in a graded series of acetone-water mixtures, and finally the blocks were embedded in Vestopal-W Ultrathin vertical cross sections of colonies were obtained with an LKB Ultrotome-III microtome and were examined either unstained or after post staining with uranyl acetate and lead citrate in JEOL JEM 100 B or a JEOL JEM 100 S electron microscope. In one experiment, colonies of *A. laidlawii* were processed for electron microscopy as described, but omitting all treatment with fixatives and stains containing heavy metals, in order to obtain unequivocal proof of the presence of tellurium deposits in the cells.

RESULTS

Among the *Acholeplasma* species investigated, colonies of *A laidlawii* and *A oculi* strongly reduced tellurite, as shown by the development of a grey coloration of the colonies on incubation with the test solution. *A. axanthum* also reduced the substrate but at a somewhat lower rate whereas *A. granularum* and *A. modicum* colonies did not show any definite signs of reduction even after 9 hours of incubation. The three *Mycoplasma* strains tested all reacted with tellurite, the two *M mycoides* strains strongly and *M bovigenitalium* to a somewhat lesser extent.

Examination of *A laidlawii* cells prepared for electron microscopy without use of heavy metals showed the presence of electron dense deposits along the periphery of cells (Fig 1) The deposits were frequently almost circular in outline. Occasionally smaller dense spots were observed in the interior of cells in areas showing very little cytoplasm (Fig 1 b) In general, the cytoplasm of cells in colonies treated with tellurite also appeared to be somewhat more contrasted (Fig. 1 c) than the cytoplasm of corresponding untreated cells which was barely visible in thin sections. Cells containing deposits of reaction products were seen along the entire surface of the colonies, wheras the central part of the colonies was essentially devoid of such cells. Moreover cells located at the circumference of a colony generally contained several distinct sites of deposit, while cells along the rest of the colony surface mostly exhibited only one (or two) reaction sites.

Peripherally localized tellurite reduction sites could be established also in *A laidlawii* cells prepared for electron microscopy by the complete procedure described above However the resulting deposits were obscured to some extent by the metals used in specimen preparation (Fig 2)

Reduction products were likewise observed at the cytoplasmic membrane in cells of *A oculi* (Fig. 3) and *A axanthum* (Fig. 4) treated with tellurite solution. In specimens of the two organisms prepared by the complete fixation and staining procedure by which membranes were well-preserved and contrasted, the electron dense deposits were seen to be located in the area between the outer and inner layer of the triple membrane (Figs. 3 and 4) and possibly also on the inner layer (Fig 3 b)

When sections of colonies of *M mycoides* subsp *mycoides* grown in the presence of tellurite were examined in the electron microscope, relatively large patches of high electron density were seen in the cytoplasm (Fig. 5) small membrane-associated deposits being observed only very rarely Cells of *M mycoides* subsp *capri* and *M bovigenitalium* treated with tellurite (Figs. 6 and 7) could not be distinguished from corresponding untreated cells.

Acta path. microbiol scand. Sect. B 85 184–188 1977

ULTRASTRUCTURAL LOCALIZATION OF TELLURITE REDUCTION IN *ACHOLEPLASMA* SPECIES

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Vinther O & Freundt, E A. Ultrastructural localization of tellurite reduction in *Acholeplasma* species Acta path. microbiol. scand Sect. B, 85 184–188, 1977

Aerobic reduction of tellurite by five *Acholeplasma* species and two *Mycoplasma* species was investigated by light and electron microscopy Among the *Acholeplasma* species, colonies of *A laidlawii* and *A oculi* exhibited a heavy macroscopically visible reduction of tellurite whereas the reaction of *A axanthum* was weaker *A granularum* and *A modicum* did not reduce the substrate under the experimental conditions employed The two subspecies of *M mycoides* also reacted with tellurite, as did also the investigated strain of *M bovigenitalium* although to a lesser extent. Ultrastructurally reduction sites were localized to the cytoplasmic membrane in the three tellurite positive *Acholeplasma* species and apparently to the cytoplasm of *M mycoides* subsp *mycoides* Reduction sites could not be demonstrated in *M mycoides* subsp *capri* and in *M bovigenitalium* The results support previous evidence obtained by biochemical methods which indicates membrane localization of redox enzymes in *Acholeplasmas*

Key words *Acholeplasma* species tellurite reduction ultrastructural localization.

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Received 1.x.76 Accepted 16.xii 76

Oxidoreductase activity is sedimented along with the membranes obtained from a number of *Acholeplasma species by osmolysis* (9 11) whereas the activity is apparently confined to the soluble fraction of *Mycoplasma* species (6 9 10 12)

It was considered of interest to investigate the cellular localization of oxidoreductases in acholeplasmas by a method not involving cell disruption and to obtain if possible a more precise picture of the sites of these enzymes. An ultracytochemical procedure utilizing the reduction of tellurite to Te as an indicator of oxidoreductase activity was used for this purpose Three *Mycoplasma* strains were included in the study for comparison.

MATERIALS AND METHODS

The following 5 *Acholeplasma* strains were tested for tellurite reduction *A laidlawii* (PG8) *A. oculi* (19-L) *A axanthum* (S743) *A modicum* (PG49) and *A granularum* (BTS-39) Also included in the study were *M mycoides* subsp. *mycoides* (PG1) *M mycoides* subsp *capri* (PG3) and *M bovigenitalium* (Kalle HE 1) All strains were cultivated on solid B medium (3) Plates were incubated aerobically for 2–6 days at 37 C, i.e until the majority of the colonies on the plates had reached sizes from 150 to 250 µm in diameter At this time 0.5 ml of a 0 1 per cent solution of K_2TeO_3

DISCUSSION

The results obtained in the present study show that tellurite reducing activity is associated with the limiting membrane in the type strains of three *Acholeplasma* species. The small deposits of reaction products observed infrequently in the interior of cells displaying little or no cytoplasm (Fig 1 b) are probably also membrane associated, being located on invaginated regions of tangentially sectioned membranes. This interpretation is in agreement with observations made by *Green & Hanson* (4). The peculiar rounded form of tellurium deposits seen in preparations where other heavy metals had not been introduced (Fig 1) may possibly be due to partial melting of tellurium (m. p. 452 C) in the electron beam caused by insufficient heat conduction in the sections. In similar studies of tellurite reduction by bacteria, needle-like deposits have been observed (14 15).

The electron dense patches in the cytoplasm of cells of *M mycoides* subsp. *mycoides* (Fig 5) are assumed to reflect sites of tellurite reducing activity since similar dense areas were not observed in cells which had not been treated with tellurite. The dark coloration of tellurite incubated colonies of *M mycoides* subsp. *capri* and *M bovigenitalium* which does not show in the electron microscope as deposits of electron dense material (Fig. 6 and 7) may perhaps be ascribed to a non-specific binding of unreduced tellurite to the cytoplasm of these cells. A general, probably non-specific increase in the contrast of the cytoplasm of *A laidlawii* cells was observed in addition to the discrete deposits (Fig. 1 c).

M gallisepticum and *M meleagridis* have previously been studied ultrastructurally after incubation with tellurite (4 7). In *M gallisepticum* crystalline deposits were observed around and inside the infra-bleb regions (7) whereas in *M meleagridis* the only detectable effect of treatment with tellurite was an increase in the contrast of the external track of the trilaminar membrane (4).

The agreement between the results of cytochemical visualization of oxidoreductase activity in *Acholeplasma* species and previous results obtained by biochemical methods following cell fractionation (9 11) makes it highly probable that the observed association with the membrane reflects the true *in vivo* localization of the electron transport chain in this genus. Furthermore, the enzyme NADH oxidase has been shown biochemically to be an integral protein of the inner track of the triple layered membrane in *A laidlawii* (8). This observation seems to agree with the presence of tellurium deposits at the inner membrane layer of one of the investigated *Acholeplasma* strains (Fig 3 b).

The method used in this investigation for light microscopic demonstration of tellurite reduction by mycoplasmas, i.e. incubation of outgrown colonies with tellurite for relatively short periods of time overcomes the problem of an inhibitory effect of the substrate sometimes encountered when tellurite is incorporated in the growth medium (1).

Fig 4 *A. axanthum* cell incubated with tellurite for 9 hours and prepared for electron microscopy by the complete fixation procedure, post-stained. Membrane localized deposits are seen (arrowheads) 113 400

Fig 5 *M mycoides* subsp *mycoides* cells incubated with tellurite for 3 hours and prepared for electron microscopy by the complete fixation procedure post-stained. Rather large and diffuse patches of high electron density assumed to represent reduced tellurite, are observed in the cytoplasm. 60,750

Fig 6 *M mycoides* subsp *capri* cells incubated with tellurite for 3 hours and prepared for electron microscopy by the complete fixation procedure, post-stained. No deposits of reduced tellurite can be observed × 74,250

Fig 7 *M bovigenitalium* cells incubated with tellurite for 9 hours and prepared for electron microscopy by the complete fixation procedure, post-stained. No deposits of reduced tellurite can be observed × 94,500



Fig 1 A laidlawii cells incubated with tellurite for 3 hours and prepared for electron microscopy without use of heavy metals, not post stained. (a) Deposits of reduced tellurite with a rounded outline are seen along the cell periphery (b) Additional small deposits are observed *in the low density areas of the cytoplasm.* (c) Besides discrete deposits along the cell periphery a general high contrast of the cytoplasm is seen. × 74 250 Bar on this and all following micrographs represents 100 nm.

Fig 2 A laidlawii cells incubated with tellurite for 3 hours and prepared for electron micro*scopy by the complete fixation procedure,* not post-stained. Small electron dense deposits are observed in the membrane area (arrowhead) × 74 250

Fig 3 A oculi cells incubated with tellurite for 3 hours and prepared for electron microscopy by the complete fixation procedure, post-stained. (a) Dense *deposits* located on the trilaminar membrane (between the two arrowheads) are observed. × 54 000 (b) Higher magnification of the part of the membrane between the arrow*heads on Fig. 3 a. Shows that deposits are loca*lised in the area between the outer and inner layer of the membrane and possibly also on the inner layer (arrowhead) × 180 000

Acta path. microbiol. scand. Sect. B, 85 189–194 1977

INTERFERON INDUCTION IN HUMAN CELL CULTURES BY SMALL MOLECULAR INDUCERS (TILORONE AND ACRIDINES)

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Degré, M. & Gláz, E. T. Interferon induction in human cell cultures by small molecular inducers (tilorone and acridines). Acta path. microbiol. scand. Sect. B, 85: 189–194, 1977.

Attempts were made to stimulate interferon production in human cell cultures by two related acridin drugs, mepacrine and Acranil. Tilorone was included for comparison. In human embryo lung fibroblasts and in normal human leukocytes only tilorone stimulated some interferon production. All three drugs stimulated modest interferon production in two lymphoblastoid cell lines. All three drugs enhanced the Sendai virus-induced interferon production in lymphoblastoid cells.

Key words: Interferon induction; human cell culture; small molecular inducers; acridin drugs.

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Received 24.xi.76 Accepted 10.xii.76

The demonstration that tilorone hydrochloride is a potent antiviral agent and interferon inducer (12, 14) has activated further research on related small-molecular weight substances for possible similar activities. During the last years several positive findings have been reported (7, 8, 9, 15, 16, 17, 18, 19, 21). Among the tricyclic compounds bearing dialkylaminoalkyl side chains which have been examined, two agents previously used as antiparasitic drugs produced antiviral effect and induced circulating interferon in mice after parenteral or oral administration. These were the related acridine drugs, mepacrine (quinacrine, Atabrin®) and Acranil® (7, 8). The present communication reports the effect of these substances *in vitro* on human cells. Tilorone was included for comparison, and some effects of this drug not previously studied were also examined.

MATERIALS AND METHODS

Cells. Human embryo lung (HEL) cell monolayers were prepared in our laboratory. The cells were grown in 50–50 per cent mixture of Eagle's Minimal Essential Medium (MEM) with Hanks salts and medium 199 with 10 per cent calf serum, and maintained in the same medium with 5 or 2 per cent serum. The cells were used for the present experiments in their 5–15th passage.

The lymphoblastoid cell lines P_3HR1 (11) and EB (6) were supplied by Dr *E. Thorsby*, Oslo. These cells were cultured as suspension cultures in Eagle's MEM with Hanks salts, supplemented with 10 per cent foetal calf serum.

Normal human leukocytes were prepared and

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TABLE 2. *Interferon Induction in Human Lymphoblastoid Cells by Mepacrine, Acranil and Tilorone*

Experiment	Interferon levels in the supernatant after stimulation with														
	Mepacrine (µg/ml)					Acranil (µg/ml)					Tilorone (µg/ml)				
	1	2	5	10	25	1	2	5	10	25	1	2	5	10	25
P IIR I cells															
1											<15	<15	28	28	90
2											<15	<15	<15	18	28
3	<15	<15	<15	56	90	<15	<15	<15	20	28					
4				28	32				28	35				32	90
EB cells															
1						<15	<15	<15	15	<15					
2						<15	<15	<15	15	18	15	28	28	90	110
3	<15	<15	<15	15	20						<15	<15	<15	20	25

Interferon units per ml.

TABLE 3. *Interferon Induction in Human Lymphoblastoid Cells by Sendai Virus and by Mepacrine, Acranil and Tilorone*

Cell type	Sendai virus	Interferon levels in the supernatant after stimulation with														
		Sendai + Mepacrine (µg/ml)					Sendai + Acranil (µg/ml)					Sendai + Tilorone (µg/ml)				
		1	2	5	10	25	1	2	5	10	25	1	2	5	10	25
P IIR I	180	780	700	1100	700	70	180	180	700	200	100	1100	700	890	890	180
EB	110	110	220	350	180	56	110	90	180	110	90	220	180	180	350	110

Interferon units per ml.

TABLE 1 *Interferon Induction in Human Embryo Lung Fibroblast Cells by Mepacrine Acranil and Tilorone*

Experiment	*Interferon levels in the supernatant after stimulation with*														
	Mepacrine					Acranil					Tilorone				
	1	2	5 (μg/ml)	10	25	1	2	5 (μg/ml)	10	25	1	2	5 (μg/ml)	10	2
1			<15*	17				<15	<15				<15	<13	
2	<15	<15	<15	<15	<15										
3				<15	<15				<15	<15				<15	
4					<15					<15					

* Interferon units per ml

cultured according to the procedure of *Cantell et al.* (2) The cells were induced with the various agents within 24 hours after bleeding. The cell concentrations in all experiments were adjusted to 10^7 per ml. The cell suspensions contained 25–40 per cent lymphocytes and 60–70 per cent polymorphonuclear leukocytes.

Viruses Vesicular stomatitis virus, Indiana strain (VSV) was propagated in the allantoic cavity of embryonated hens eggs and then passaged twice in L-F1 mouse fibroblast cells. Infectivity titre was assayed by the infectivity end point micromethod on *L-F1 cells. Parainfluenza 1 virus*, Sendai strain was grown in the allantoic cavity of embryonated hens eggs and titrated by haemagglutination using guinea pig erythrocytes. Pools of both viruses were kept at −?1 C.

Chemicals Acranil dihydrochloride and mepacrine dihydrochloride were supplied by Bayer AG Elberfeld, Germany and tilorone dihydrochloride was a gift from Dr *R F Krueger* The Merrell National Laboratories, Cincinnati Ohio USA Fresh solutions in physiological saline were made for each experiment.

Interferon assay Supernatants from the induced cell cultures were adjusted to *pH 2 by 1N HCl* After 2 days the samples were neutralized by NaOH. Interferon titres were estimated by a microinfectivity inhibition test (3) that used HEL *cells and* VSV The titres are expressed in international standard units (69 19 reference sample)

RESULTS

Toxicity in Cell Cultures

HEL cell monolayers were grown in the presence of various *concentrations of* the three drugs. The cells were examined daily for signs of toxicity by light microscopy No morphological alterations were seen in the presence of concentrations up to 5 μg/ml. Slight toxicity was observed after 4-5 days on cells treated with 10 and 25 μg/ml of mepacrine and *Acranil* and with 25 μg/ml of tilorone Higher concentrations of all three drugs were markedly toxic within two days.

Toxicity on lymphoblastoid cells P_3HR1 was also tested. Viability of the cells after 3 days of incubation with various concentration of the drugs was tested by the trypan blue *exclusion method. More than* 95 per cent of the cells were viable in the presence of up to 5 μg/ml of any of the three drugs. Sixty per cent were viable in the presence of 10 and 40 per cent in the presence of 25 μg/ml of mepacrine of Acranil. The toxic effect of tilorone was comparable or possibly *slightly lower*

Interferon Induction in HEL Cells

Three to five day old monolayers of HEL cells were incubated in medium containing various concentrations of the three drugs. The supernatants were collected after 20 hours and assayed *for interferon* activity In some experiments the inducers were removed after 2 hours, and the cells were replenished with fresh medium and incubated for 20 hours. There was no apparent difference between the two different types of stimulation and therefore only the first type of experiments are presented in Table 1 Neither Acranil, nor mepacrine induced detectable amounts of interferon. On the other hand, tilorone induced modest but significant titres

inducing effect of tilorone has also been observed with other cells (5)

Several facts indicate that following *in vivo* stimulation with small-molecular inducers the main site of interferon production is in the lymphoid tissue. Highest concentrations were found in organs such as thymus, spleen, and in some experiments lung (1 4 17). Although *Stringfellow & Glasgow* (20) could not reduce the tilorone-induced interferon levels by antilymphocyte serum in mice, this was achieved in X irradiated animals. *Dennis et al.* (5) demonstrated clearly in *in vitro* experiments that human lymphocytes stimulated by this drug do produce interferon. This has been confirmed in the present study using established lymphoblastoid cell lines and apparently non-toxic concentrations of tilorone. Interferon was also produced under similar conditions by human leukocytes. The lymphocyte population is probably the most active interferon producer in this case. Acranil and mepacrine induced modest interferon production in the lymphoblastoid cells, but only in slightly toxic concentrations. When non-toxic concentrations of these two drugs were applied in leukocyte cultures, no interferon could be found.

All three drugs enhanced the Sendai virus-induced interferon production in lymphoblastoid cells. This observation is an extension of the findings of *Groelke et al.* (10) who reported a synergistic interferon induction by tilorone and polyriboinosinic-polyribocytidylic acid in murine cells. It is possible that the interferon stimulation in the lymphoblastoid cells by the drugs alone is also an enhancement effect. Established lymphoblastoid cell lines usually contain several copies of Epstein-Barr virus genom. Spontaneous interferon production has been demonstrated in several such lines (13). Although we could not find spontaneous production in our lines, it might have been present in small amounts not detectable by the method employed. The lack of interferon production by the acridine drugs in normal leukocyte cultures also supports this notion.

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of interferon when added in a moderately toxic concentration. The antiviral activity was characterized as interferon by the standard criteria. Direct addition of the drugs did not influence the multiplication of VSV.

Interferon Induction in Lymphoblastoid Cells

Suspensions of P_3HR1 and EB_4 cells, containing ca. 10^7 cells per ml were supplemented with Acranil, mepacrine or tilorone in various concentrations. In some experiments the inducers were removed after 2 hours and the cells were replenished with fresh medium. In others the cells were incubated in the presence of inducers for the entire period of 20 hours. Then the cells were sedimented by centrifugation at 3000 rev / min for 10 minutes, and the supernatants were tested for interferon activity (Table 2). All three drugs induced interferon production in both cell types, although the titres were generally low. The production was to some extent dose-dependent. The effective doses of the two acridines were partially toxic. Removal of the inducers after 2 hours did not influence the interferon titres.

Double Induction in Lymphoblastoid Cells with Viral and Chemical Inducers

To suspensions of P_3HR1 or EB_4 cells, containing 10^7 cells per ml, 20 per cent (v/v) Sendai virus 600 haemagglutinating units per ml, and an equal volume of medium containing various concentrations of the three drugs were added. After incubation for 20 hours the suspensions were clarified by centrifugation and the supernatants were tested for interferon activity (Table 3). All three drugs enhanced the interferon production in both cell types, as compared to the titres induced by the Sendai virus alone. Maximal effect of mepacrine and Acranil was seen with the highest perceptibly non-toxic concentration 5 μg/ml. No clear dose-dependency could be seen for tilorone. Toxic concentrations reduced the interferon production in all experiments.

Interferon Induction in Normal Human Leukocytes

To leukocyte suspensions containing 10^7 cells per ml, 20 per cent (v/v) Sendai virus, 600 haemagglutinating units per ml, or an equal volume of medium containing tilorone, Acranil or mepacrine were added to final concentrations of 5 and 10 μg/ml. The cultures were incubated in a roller for 20 hours, then the cells were sedimented by centrifugation at 3000 rev /min for 10 minutes and the supernatants tested for interferon activity. The Sendai virus infectivity was destroyed by HCl treatment at pH 2 for 2 days at 4° C. The Sendai virus-induced leukocytes produced 2–6000 units per ml. Tilorone-induced cultures contained marginal activity (15–20 units per ml) while the other drugs did not induce production of detectable amounts of interferon.

DISCUSSION

The small-molecular weight interferon inducers seem to have numerous common biological characteristics, in spite of structural differences. They all provide antiviral activity and induce interferon production *in vivo* in mice and in some other experimental animals. The kinetics of interferon production are typically delayed and prolonged (4, 15, 17, 20, 21).

Attempts to induce interferon production *in vitro* in non immunocompetent monolayer cell cultures were largely unsuccessful (4, 5, 10, 15, 17, 20). In our experiments Acranil and mepacrine did not stimulate detectable interferon levels in HEL cultures, but when added in moderately toxic doses, tilorone did. It might be worth mentioning that treatment of mouse embryo fibroblasts with the same concentration of tilorone as in our experiments stimulated interferon production (10). The same authors failed to produce interferon in human embryo fibroblasts. However they used lower concentration of the drug in these negative experiments (10). Correlation between the toxic effect and the interferon-

Acta path. microbiol. scand. Sect. B, 85: 195–200, 1977

INDIRECT IMMUNOFLUORESCENCE DETECTION OF HUMAN IgM AND IgG ANTIBODIES AGAINST HERPES SIMPLEX VIRUS TYPE 1 INDUCED CELL SURFACE ANTIGENS

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Marttila, R. J. & Kalimo, K. O. K. Indirect immunofluorescence detection of human IgM and IgG antibodies against herpes simplex virus type 1 induced cell surface antigens. Acta path. microbiol. scand. Sect. B, 85: 195–200, 1977.

An indirect immunofluorescence method, based on the use of infected HeLa cell coverslip cultures was developed to demonstrate human IgM and IgG class antibodies against herpes simplex virus (HSV) type 1 induced cell surface antigens. A total of 35 specimens from 20 patients have been tested, including patients with a clinical diagnosis of HSV type 1 or type 2 primary infection, patients with recurrent HSV infections, patients without any HSV infections, and patients with varicella-zoster virus (VZV) infections. In each patient with a primary HSV infection both IgM and IgG antibody response was observed, while the patients with recurrent HSV infections showed only IgG antibodies. The direct serological typing of HSV infections was not possible because of cross-reacting antibodies both in the IgG and IgM test. No cross-reactivity was found in this test with HSV and VZV antibodies. The HSV fluorescent IgG and IgM antibody titers were found to parallel the highly sensitive HSV radioimmunoassay antibody titers very closely though at a markedly lower level.

Key words: Immunofluorescent detection; IgG antibody; IgM antibody; herpes simplex virus; cell surface antigens.

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Received 17.xi.76 Accepted 20.xii.76

Herpes simplex virus infected cells acquire virus-specific antigens on their cytoplasmic membranes. These antigens have been demonstrated by several methods, e.g. immunofluorescence (3, 9, 11, 20), complement dependent cell lysis (12, 19), mixed absorption (2, 4) or antibodies tagged to ferritin (15). The HSV induced cell surface antigens have been found to correspond to components of the viral envelope in antigenic specificity as well as in biochemical properties (12, 13, 14).

Immunofluorescent techniques for the detection of human antibodies to HSV induced surface antigens have employed infected cells in suspension (9, 20). However, in terms of manipulations required, these techniques are laborious to carry out.

We here report a simple, indirect immunofluorescent method based on the use of in-

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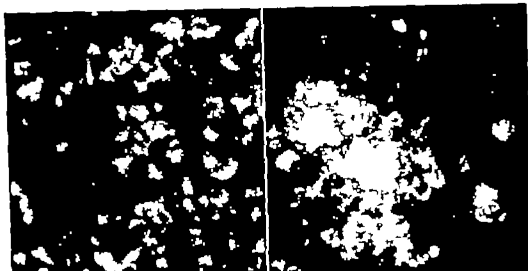


Fig. 1. Immunofluorescence detection of IgG antibodies against HSV type 1 induced cell surface antigens. Left: negative serum; right: positive serum.

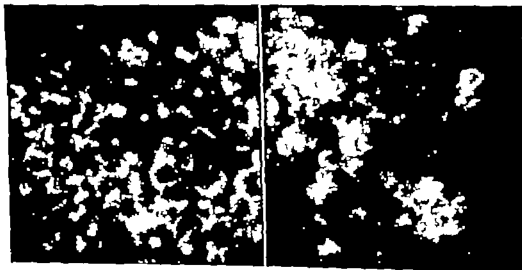


Fig. 2. Immunofluorescence detection of IgM antibodies against HSV type 1 induced cell surface antigens. Left: negative serum; right: positive serum.

In the specimens from each patient with a clinical diagnosis of HSV primary infection (Nos. 1–9) a fourfold or greater increase in IgG antibodies was observed in the FAT during the convalescent phase of the disease. No differences were found between patients with presumable type 1 infection (stomatitis and keratitis) and those with type 2 infection (genital herpes). Generally FAT IgG titers paralleled the corresponding RIA titers, though at a one to two hundred times lower level.

In patients with a primary HSV infection a definite IgM response was found in the

fected coverslip cultures in the demonstration of both IgM and IgG class antibodies in human sera to HSV induced surface antigens.

MATERIALS AND METHODS

Cells Hela Bristol cells were used throughout the study. Cells grown in 120 cm² Roux bottles were detached with 0.2 per cent trypsin—0.02 per cent EDTA solution, dispersed and seeded on coverslips in the bottom of 150 mm diameter Petri dishes, each dish containing 5 coverslips. Eagle's minimum essential medium supplemented with 10 per cent of foetal calf serum and 5 per cent of tryptose phosphate broth was used as culture medium. After an overnight growth the cultures usually showed a confluent monolayer.

Virus The VR strain of HSV type 1 was used at a multiplicity of 5–10 PFU per cell to infect the coverslip cultures. Before infection the cultures were washed three times with Hanks' balanced salt solution. After 1 hour adsorption of virus inoculum at 25 C the maintenance medium Eagle's basal medium with 0.2 per cent of bovine serum albumin was added. The cultures were incubated at 37 C for 6 hours for IgG antibody tests and for 10 hours for IgM antibody tests.

Immunofluorescent technique After washing the cultures three times with phosphate buffered saline (PBS) they were allowed to dry at room temperature for 5–10 minutes. The surfaces of the coverslips were divided into 12 squares with nail polish to prevent the serum dilutions from mixing together. Without any fixation twofold serial serum dilutions (inactivated for 30 minutes at 56 C) were applicated in each square and incubated in a moist chamber at 37 C for 45 minutes in IgG tests and for 3 hours in IgM tests. After washing with three changes of PBS the FITC-conjugated sheep anti-human IgG (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) or sheep anti-human IgM (Wellcome, Beckenham, England) was added in optimal working dilutions, checkerboard titrated before hand. After incubation in a moist chamber at 37 C for 45 minutes the coverslips were washed as above. The specimens were mounted with buffered glycerol pH 8.5 and examined with a Leitz Ortholux fluorescence microscope. The endpoint titer of a serum specimen was defined as that serum dilution which still showed a definite specific membrane fluorescence.

HSV radioimmunoassay (RIA) A solid phase radioimmunoassay developed in our laboratory for detection of IgM and IgG class antibodies against HSV was used as previously described in detail (5).

Briefly fourfold serial serum dilutions were incubated with the antigen coated polystyrene balls. After 1 hour incubation at 37 C the serum was aspirated off and the balls were washed twice with 5 ml of tap water. Aliquot of ^{125}I labeled anti-human μ or γ immunoglobulins containing 30,000 cpm was added to each tube. After incubation for 1 hour at 37 C the radioactive solution was aspirated off and the balls were washed as above. The radioactivity bound to the balls was counted with a LKB Wallac gamma counter. Endpoint titer values were obtained from the cpm versus dilution curve (log 2) of each specimen. Titers were expressed as log 2 values of reciprocals of the serum dilutions.

Serum specimens Altogether 35 specimens from 20 patients were included in the study. Paired serum samples were obtained from 6 patients with a clinical diagnosis of HSV type 1 primary infection and from 3 patients with a genital primary HSV infection. Samples from 5 patients with recurrent HSV infections and from 2 patients with no history of HSV infection were included. Paired serum samples from 2 patients with clinical varicella and from 7 patients with herpes zoster were also tested.

RESULTS

Preliminary experiments Tests with known HSV antibody negative and positive sera were carried out in order to establish the optimum conditions for the fluorescent antibody test (FAT). Different incubation times (4, 6, 10, 18 hours) of cultures with virus were tested. Optimal results (bright specific fluorescence and absence of unspecific fluorescence) were observed after 6 hours incubation in the IgG test and after 10 hours incubation in the IgM test respectively. The multiplicity of infection was also tested and the best results were obtained with 5–10 PFU per cell.

Sera negative for HSV IgG antibody in RIA gave no specific fluorescence in the IgG FAT. A clear membrane fluorescence was observed with sera positive for HSV IgG antibody in RIA (Fig. 1). In the IgM FAT the specific fluorescence was less intense and the background fluorescence was somewhat more apparent (Fig. 2). However specific fluorescence was easily recognized.

Antibody assay results The results of testing the patients' sera are shown in Table 1 in comparison with the RIA HSV antibody titers.

using infected coverslip cultures in the FAT. We have used this method for human serum specimens and, in addition, to the demonstration of IgM and IgG class antibodies to HSV induced surface antigens.

Human HSV IgM antibodies have been detected by indirect immunofluorescence using both fixed (7 18) and unfixed infected cells (9 20) but the methods are rather laborious and difficult to carry out in testing a large number of serum specimens. Using fixed cells absorption of serum specimens with tissue culture cells is often necessary because of unspecific fluorescence. To increase the IgM specific fluorescence testing serum IgM fractions after sucrose density gradient centrifugation (7) or testing sera after removal of IgG by *Staphylococcus aureus* absorption (18) have been used. In detecting IgM antibodies against surface antigens in HSV infected cell suspensions several centrifugations are necessary during the immunofluorescent staining procedures (9 20).

In our fluorescent antibody method HSV infected unfixed coverslip cultures are used and HSV IgM and IgG antibody detection is possible without any treatment of the serum specimens except heat inactivation. However it is to be expected that antiimmunoglobulins, such as rheumatoid factor will interfere with the IgM test described as also with the other FATs in the IgM antibody detection (10 17). Compared to the RIA IgM and IgG antibody titers, the FAT titers were lower but in no case would the diagnosis of recent HSV infection have been lossed.

The multiplicity of infection is one of the important factors in the test. Cells showing specific fluorescence should be properly scattered so that the interpretation of the test is straightforward and reliable. The incubation time of the cultures with virus is also important. For the IgG test the incubation time of 6 hours was found to be optimal. Increasing the time results in an increase of unspecific fluorescence possibly related to the induction of Fc receptors on the cell surfaces of HSV infected cells (22) receptors which bind other immunoglobulins than HSV antibodies. However for the IgM test a 10 hours incubation time was found to be necessary even if it brought along some unspecific staining.

Since the host antibody response is predominantly directed against the envelope components of HSV (1 8) concerning both IgM and IgG response (6) tests detecting antibodies against these antigens are particularly useful in primary HSV infections. The surface antigens have been found to resemble the components of HSV envelope (13 14). These antigens appear on the cytoplasmic membranes shortly after infection reaching their maximum at 8 to 10 hours postinfection (4 15). The eclipse time of HSV infection in Hela cells is found to be about 12 hours, and respectively the latent period is up to 16 hours (21). Consequently it seems that within the time schedule of the present FAT the other HSV antigens do not interfere with the surface antigens, the test thus measuring antibodies mostly against HSV surface antigens.

A disadvantage of this method is that direct serological typing of HSV infections is not possible, since HSV type 1 and 2 share common envelope antigens inducing both IgM and IgG cross-reacting antibodies (1 6 8). On the other hand, no cross-reactivity was found with VZV antibodies, as also found when studying antibodies to surface antigens induced by VZV (23). This cross-reactivity is commonly seen in the complement fixation test and in immunofluorescent test with fixed antigens (16). However some cross-reactivity has been found in RIA between HSV and VZV antibodies, even with the envelope antigen of HSV (*Kalimo K.* unpublished observations) suggesting the absence of cross-reactivity in the surface antigen FAT is partly due to the lower sensitivity of this technique.

This study was supported by the *Academy of Finland* *Medical Research Council*. The excellent technical assistance of Mrs. *Leena Nurmi* and Mrs. *A ja K pi* is gratefully acknowledged.

TABLE 1 *FAT IgG and IgM Titers against HSV Type 1 Induced Surface Antigens in Comparison to HSV RIA IgG and IgM Antibody Titers. All Titers Are Expressed as Log 2 Values*

Clinical diagnosis	Age in years	Days after onset	IgG FAT	IgG RIA	IgM FAT	IgM RIA
1 stomatitis	1	2	<3	<4	<2	<4
		14	9	15	5	12
2 stomatitis	2	4	<3	<4	<2	<4
		18	10	15	6	11
3 stomatitis	4	6	<3	<4	<2	10
		24	10	14	3	12
4 stomatitis	14	4	<3	8	<2	8
		15	9	15	6	13
5 keratitis	15	3	<3	<4	<2	<4
		15	10	16	5	12
6 stomatitis	17	6	<3	9	3	11
		11	9	15	4	13
7 genital herpes	17	5	<3	6	<2	9
		18	7	14	3	12
8. genital herpes	23	10	4	10	<2	7
		25	8	12	4	7
9 genital herpes	27	7	<3	<4	<2	<4
		22	7	14	4	12
10 recurrent herpes	25	1	8	14	<2	<4
11 recurrent herpes	27	2	7	15	<2	<4
		16	8	14	<2	<4
12. recurrent herpes	31	7	8	12	<2	<4
13. recurrent herpes	37	3	8	14	<2	<4
14 recurrent herpes	46	8	7	15	<2	<4
15. no herpes history	28		<3	<4	<2	<4
16. no herpes history	31		<3	<4	<2	<4
17 varicella	12	1	<3	<4	<2	11
		3	<3	9	<2	<4
18. varicella	20	1	<3	<4	<2	<4
		15	<3	<4	<2	9
19 herpes zoster	56	3	7	13	<2	9
		11	7	13	<2	5
20 herpes zoster	81	7	6	11	<2	6
		19	7	13	<2	

FAT with titers one to two hundred times lower than by RIA As a consequence three acute phase specimens (Nos. 3 4 7) positive in RIA were negative in the FAT However FAT demonstrated an IgM antibody response also in these patients

All specimens taken from patients with recurrent HSV infections (Nos. 10–14) were positive for IgG antibodies both in the FAT and in RIA, whereas IgM class antibodies were not observed in either test.

Serum specimens from two patients with out any history of HSV infections (Nos. 15 16) and negative in RIA, were also negative in the FAT

Apparent cross-reacting HSV antibody activity was found by RIA both in IgG and IgM tests in specimens from patients with varicella-zoster virus infections (Nos. 17 19 20) while in the HSV FAT no cross-reactivity was observed

DISCUSSION

Geder & Skinner (3) detected antibodies to HSV induced surface antigens in animal sera

Acta path. microbiol. scand. Sect. B, 85: 201-211, 1977

ULTRASTRUCTURAL STUDIES ON THE ENDOGENOUS DEVELOPMENT OF *EIMERIA BRUNETTI*

IV. *Formation and Structure of the Oocyst Wall*

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Ferguson, D. J. P., Birch-Andersen, A., Hutchison, W. M. & Siim, J. Chr. Ultrastructural studies on the endogenous development of *Eimeria brunetti*. IV. Formation and structure of the oocyst wall. Acta path. microbiol. scand. Sect. B, 85: 201-211, 1977.

The ultrastructural changes occurring during wall formation in the oocysts of *E. brunetti* were studied in tissue from the small intestine of young domestic fowls. The process of oocyst wall formation was initiated by a separation of the limiting membranes of the cytoplasmic mass of the macrogamete, to form a loose veil, consisting of 2-3 membranes, around the organism. Concomitant with the appearance of the veil, changes were observed in the Wall Forming Bodies of Type I (WFB I) and Type II (WFB II) which were present in the cytoplasm of the macrogamete. The outer layer of the oocyst wall seemed to be formed by a combination of membranes released from the surface of the organism and the contents of the WFB I. A 700 nm thick layer was thereby formed and, at this stage, the WFB I had disappeared from the cytoplasmic mass. The outer layer further condensed into a structure 250–350 nm in thickness in which 3 zones of different densities could be distinguished. The formation of the inner layer of the oocyst wall appeared to be associated with material from the WFB II and these disappeared from the cytoplasmic mass as the inner layer became evident. The inner layer was homogeneous in appearance and 80 nm thick. Comparison of the ultrastructure of the walls of oocysts obtained from chicken faeces with that of oocysts observed within the intestine showed only minor differences. The loose veil was not present in oocysts isolated from faeces and some changes were also noted in the outer zone of the outer layer of the oocyst wall. These differences could result from the mechanical and chemical treatments used for processing the faeces.

Key words: *Eimeria brunetti*; chickens; oocyst wall formation; ultrastructure.

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Received 16.x.76 Accepted 28.I.77

* Work initiated while a Wellcome Trust Travelling Research Fellow and completed as a Danish Medical Research Council Fellow.

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1

WFBI

PV

LM

AL

PG

WFBI

WFBII

WFBII

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4

UM

WFBII

WFBI

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LM

In the coccidia, the oocyst is the form of the organism which must survive in the external environment in order to provide a means of transmission of the parasite from one host to another. To protect the oocyst against the rigors of the external environment it has a protective wall which is formed prior to the organism leaving the host cell and being shed with the faeces of the infected host.

In previous reports we have published details of the ultrastructural aspects of schizogony, microgametogony and macrogametogony in *Eimeria brunetti* – a pathogenic coccidian of domestic fowls (5, 6 & 7). In this paper we will describe the ultrastructural changes which were found to occur as the wall was formed around the developing oocyst. In addition the sub-structure of the walls of oocysts still present within the epithelial cells of the host will be compared to that seen in oocysts which had been excreted with the faeces.

MATERIALS AND METHODS

The techniques used in the present study to examine the endogeneous development have been previously described (5) but can be summarised as follows. The chickens were killed at 120 and 144 hours post infection (p.i.). Pieces of the small intestine containing parasites were fixed in glutaraldehyde and osmium tetroxide and embedded in Vestopal W. Thin sections were examined in the electron microscope after staining with magnesium uranyl acetate and lead citrate. The observations on oocyst wall formation were made on material from the same series of chickens as used in our previous studies and were based on the examination of approximately 400 micrographs.

The techniques involved in the examination of the ultrastructure of the wall of oocysts obtained from chicken faeces have been previously described (1) but can be summarised as follows. Oocysts were pre-embedded in cross linked bovine serum albumin (BSA), sectioned with a cryostat after rapid freezing in liquid nitrogen and fixed in Karnovsky's fixative (9) followed by osmium tetroxide. The final embedding, sectioning, staining and examination was carried out as summarised for the infected tissue.

RESULTS

Macrogametes on which signs of oocyst wall formation were obvious were only observed in chickens killed at 120 and 144 hours p.i.

The ultrastructural details of the macrogamete were as previously described (7). It is believed that fertilization of the macrogamete by a microgamete occurs prior to oocyst wall

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Figures 1–13 are all electron micrographs obtained from sections of epithelial cells of the small intestine of chickens infected with *E. brunetti* and illustrate the formation and structure of the oocyst wall.

A double bar (=) on a micrograph represents 1 µm and a single bar (—) represents 100 nm.

The following abbreviations are used throughout: AL – Amorphous Layer. C – Canaliculi. CM – Cytoplasmic Mass of the Oocyst. ER – Rough Endoplasmic Reticulum. I – Inner Layer of the Oocyst Wall. LM – Limiting Membrane. MI – Mitochondrion. MP – Micropore. N – Nucleus. NU – Nucleolus. O – Outer Layer of the Oocyst Wall. PG – Polysaccharide Granule. PV – Parasitophorous Vacuole. UM – Unit Membrane. V – Loose Veil surrounding the Oocyst. WFB I – Wall Forming Bodies of Type I. WFB II – Wall Forming Bodies of Type II. 1 – Granular Zone of the Outer Layer of the Oocyst Wall. 2 – Homogeneous Zone of the Outer Layer of the Oocyst Wall. 3 – Osmophilic Zone of the Outer Layer of the Oocyst Wall.

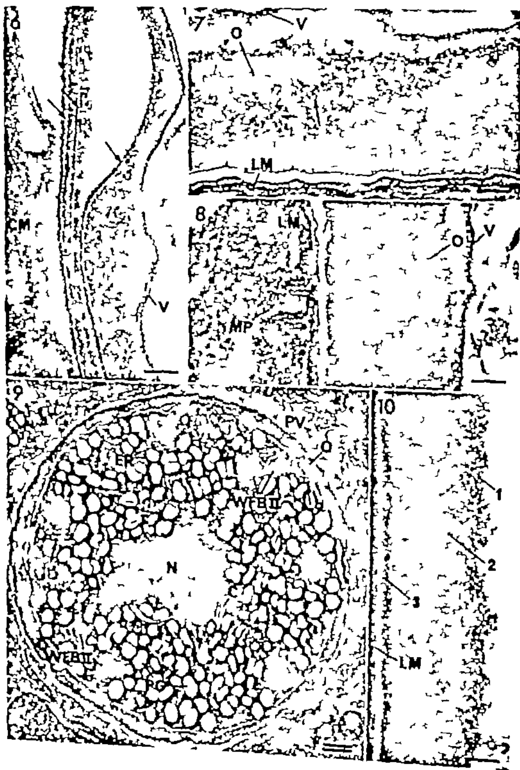
Fig. 1. A very early stage in oocyst formation is illustrated. At this stage the organism can be seen to be surrounded by a loose veil (arrows). The cytoplasm contains polysaccharide granules, mitochondria, canaliculi, WFB I and WFB II. The nucleus is not included in this section. × 7,500.

Fig. 2. Part of a section through the periphery of an organism showing the two closely applied unit membranes below the limiting membrane. An amorphous layer is present on the exterior of the limiting membrane. Note the separation of the two unit membranes (arrow) which is thought to represent the first indication of the veil formation. × 160,000.

Fig. 3. Part of the periphery of an organism similar to that in Fig. 1. In this region three membranes of the loose veil (arrows) can be seen exterior to the limiting membrane of the organism. × 90,000.

Fig. 4. A higher magnification of part of Fig. 1. A dissolving WFB I is shown in which it would appear that the homogeneous material passes through a granular phase (arrow) before being replaced by a more electron translucent material. × 45,000.

Fig. 5. Part of a section of an organism similar to that in Fig. 1 showing the sponge-like appearance of the WFB II at this early stage of oocyst development. × 45,000.


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formation. In our material however although *microgametes* were seen in the cytoplasm of host cells which contained *macrogametes*, no evidence for a fusion of these gametes was observed.

The macrogamete was surrounded by an amorphous layer below which a pellicle was present. The pellicle consisted of a single outer unit membrane plus two closely applied inner unit membranes. The first evidence of the initiation of oocyst development was a separation of the two closely applied inner membranes (Fig. 2). The amorphous layer and the two other membranes had in this way become separated from the cell surface and a loose veil surrounding the organism was formed (Fig. 1). This veil appeared to be made up of two or three membranes of variable thickness (50–120 nm) none of which presented a typical unit membrane structure (Fig. 3). The organisms at this stage possessed the organelles previously described as being present in the macrogamete viz. a nucleus with its nucleolus, multi membranous vacuoles, polysaccharide granules, canaliculi, mitochondria and wall forming bodies of Type I and Type II (WFB I & WFB II) (Fig. 1). In both types of WFB some structural changes were observed at this early phase of oocyst formation (Fig. 1). In the WFB I it appeared that the electron dense contents had started to dissolve as evidenced by changes in staining characteristics, although the shape, size, and limiting membrane were still retained (Fig. 4). The electron dense material seemed to pass through a transitional phase which was granular in appearance before being replaced by an electron translucent material observed in the peripheral region of the granule (Fig. 4). In the WFB II the homogeneous appearance was also lost and they took on a sponge like appearance (Fig. 5). It was common to find 2 or 3 WFB II within the same cisterna of the rough endoplasmic reticulum (Figs. 1 & 5).

In the formation of the outer layer of the oocyst wall a continuous synthesis and release of membranes from the surface of the organism seemed to be involved. The membranes were apparently coated with material from the WFB I and a laminated structure was formed within which the unit membranes could no longer be distinguished (Fig. 6). The layer was initially uneven in thickness but appeared to become of uniform thickness (approx. 700 nm) in more developed oocysts (Figs. 8 & 9). In these the outer layer consisted of homogeneous material with some granular material present in certain regions (Fig. 7). The material resembled the contents of the dissolving WFB I both in appearance and staining properties (cf. Figs. 4 & 7). When the formation of the outer layer was complete, the WFB I had disappeared from the cytoplasm of the organism and only a number of small vesicles remained (Fig. 9). Up to this stage the other cytoplasmic organelles remained unaffected (Fig. 9). It should also be noted that it was possible to observe

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Fig. 6. Part of the periphery of an organism in which the outer layer of the oocyst wall appears to be in the process of formation. Note that the membranes seem to be released and that they are coated with homogenous material (arrows). × 90,000.

Fig. 7. Part of the periphery of an organism similar to that in Fig. 9. At this stage of development the outer layer of the oocyst wall is homogeneous in appearance with a few patches of granular material (arrow). × 90 000.

Fig. 8. Part of the periphery of an organism similar to that in Fig. 9. A micropore with typical substructure is present on the limiting membrane of the cytoplasmic mass below a well developed outer layer of the oocyst wall. × 90 000.

Fig. 9. A section through an organism in which the outer layer of the oocyst wall is of uniform thickness. The cytoplasm contains a nucleus, numerous polysaccharide granules, canaliculi and a few strands of rough endoplasmic reticulum. At this stage of wall formation the inner layer is not formed and the WFB II are still present. Note that remnants of WFB I are present as small vesicles (arrows). × 7,500.

Fig. 10. Part of the periphery of an organism in which the outer layer of the oocyst wall has condensed into a structure exhibiting three distinct zones, i.e. an outer granular zone, a middle homogeneous zone and an inner osmiophilic zone. The inner layer has not been formed. × 90 000.

micropores on the limiting membrane of the cytoplasmic mass below the forming oocyst wall (Fig 8)

Before the formation of the inner layer of the oocyst wall, the WFB II seemed to have become still less homogeneous in appearance than those present in less developed oocysts

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Fig 11 A portion of an organism showing the initiation of formation of the inner layer of the oocyst wall. A membrane (arrow) is observed below the outer layer of the oocyst wall. Between this membrane and the limiting membrane of the cytoplasmic mass, patches of material (large arrow) similar in appearance to the contents of the WFB II are found. The outer layer is unusually thin in this organism. × 45,000.

Fig 12 Part of the periphery of a fully developed oocyst showing the architecture of the wall. The oocyst wall consists of a loose veil, outer layer (differentiated into 3 zones) and a homogeneous inner layer. The cytoplasmic mass of the oocyst is limited by a unit membrane and has shrunk away from the wall. The space thus formed is filled with a finely dispersed, moderately dense material. The electron translucent region between the outer and inner layers of the oocyst wall is probably an artifact because of improper penetration or polymerization of the embedding medium. × 90,000

Fig 13. An organism with a fully formed oocyst wall. The cytoplasm contains a nucleus with a distinct nucleolus, a number of polysaccharide granules and few remnants of the WFB II (arrow). The irregular shape of the oocyst is believed to be an artifact caused by slight shrinkage because the oocyst wall represents major barrier to fixatives and other reagents × 7,500

Figures 14-16 are electron micrographs which illustrate the structure of the walls of oocysts obtained from chicken faeces. The loose veil seen around oocysts present in the mucosa of the host is absent in this material. In these oocysts, the wall consists of an outer layer which could be differentiated into 3 zones and an inner homogeneous layer. Variations are observed in the granular zone of the outer layer of the oocyst wall. The material present exterior to the outer oocyst wall is the cross linked BSA used for primary embedding.

Fig 14 The granules form zone of nearly uniform thickness. × 90,000

Fig 15 The granules are present in aggregates or clusters × 90,000

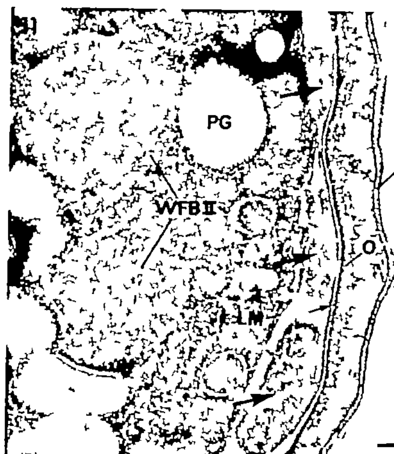
Fig 16 The granules are absent. × 90,000.

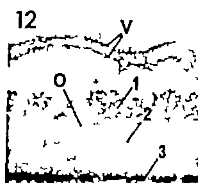
(Fig. 11) A region of less electron dense material was often observed around the WFB II present in the cisternae of the rough endoplasmic reticulum (Fig 9) The first evidence of formation of the inner layer of the oocyst wall was presented with the appearance of an approximately 15–20 nm thick membrane below the outer layer of the oocyst wall (Fig. 11) Between this membrane and the limiting membrane of the organism, patches of sponge like material similar in appearance to that observed within the WFB II were seen (Fig 11) It appeared that these patches could fuse and thus the inner layer of the oocyst wall was formed (Fig 12)

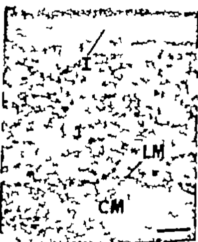
Once the complete oocyst wall was formed, the organism was found to contain a centrally located nucleus with a prominent nucleolus. The cytoplasm was densely packed with polysaccharide granules but a few mitochondria and some strands of rough endoplasmic reticulum were also present (Fig. 13) The WFB I was not observed but a few remnants of the WFB II could still be distinguished (Fig 13) After the maturation of the oocyst wall, the cytoplasmic mass of the organism appeared to be reduced in size and it was always limited by a single unit membrane (Fig. 12) It also seemed to have shrunk away from the oocyst wall and a finely dispersed moderately dense material filled the region between the oocyst wall and the cytoplasmic mass (Fig 12)

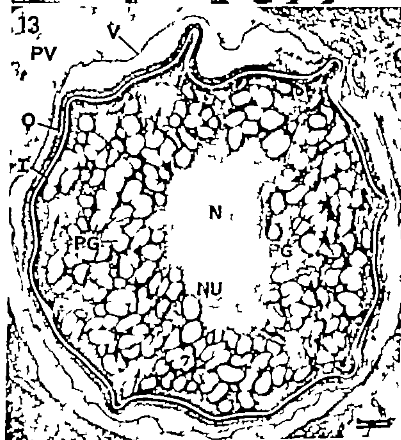
Sub-structure of the fully formed oocyst wall The fully formed oocysts, that were present within the host epithelial cells, always possessed the delicate veil (Fig 12) Under the veil, the wall proper was made up of two well defined layers.

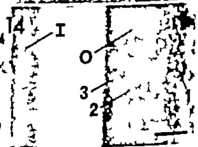
The outer layer of the oocyst wall appeared as a homogeneous layer 700 nm in thickness in the earlier stages of wall formation but was found condensed, in later stages, into a layer approximately 250–350 nm thick. When fully formed, this layer could be differentiated into three zones (Figs. 10 & 12) The outer zone had a granular appearance and varied between 70–140 nm in thickness. The middle zone had a homogeneous appearance and varied between 150–200 nm in thickness. The

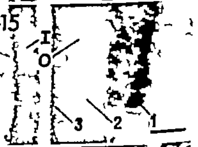

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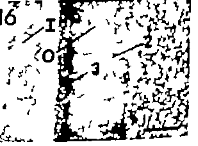

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micropores on the limiting membrane of the cytoplasmic mass below the forming oocyst wall (Fig 8)

Before the formation of the inner layer of the oocyst wall, the WFB II seemed to have become still less homogeneous in appearance than those present in less developed oocysts (Fig 11) A region of less electron dense material was often observed around the WFB II present in the cisternae of the rough endoplasmic reticulum (Fig 9) The first evidence of formation of the inner layer of the oocyst wall was presented with the appearance of an approximately 15–20 nm thick membrane below the outer layer of the oocyst wall (Fig. 11) Between this membrane and the limiting membrane of the organism, patches of sponge like material similar in appearance to that observed within the WFB II were seen (Fig 11) It appeared that these patches could fuse and thus the inner layer of the oocyst wall was formed (Fig. 12)

Once the complete oocyst wall was formed, the organism was found to contain a centrally located nucleus with a prominent nucleolus. The cytoplasm was densely packed with poly saccharide granules but a few mitochondria and some strands of rough endoplasmic retic ulum were also present (Fig 13) The WFB I was not observed but a few remnants of the WFB II could still be distinguished (Fig. 13) After the maturation of the oocyst wall, the cytoplasmic mass of the organism appeared to be reduced in size and it was always limited by a single unit membrane (Fig 12) It also seemed to have shrunk away from the oocyst wall and a finely dispersed moderately dense material filled the region between the oocyst wall and the cytoplasmic mass (Fig 12)

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Fig 11 A portion of an organism showing the initiation of formation of the inner layer of the oocyst wall. A membrane (arrow) is observed below the outer layer of the oocyst wall. Between this membrane and the limiting membrane of the cytoplasmic mass, patches of material (large arrow) similar in appearance to the contents of the WFB II are found. The outer layer is unusually thin in this organism. × 45,000.

Fig 12 Part of the periphery of a fully developed oocyst showing the architecture of the wall. The oocyst wall consists of a loose eil, outer layer (differentiated into 3 zones) and a homogeneous inner layer The cytoplasmic mass of the oocyst is limited by unit membrane and has shrunk away from the wall. The space thus formed is filled with finely dispersed, moderately dense material The electron translucent region between the outer and inner layers of the oocyst wall is probably an artifact because of improper penetration or polymerization of the embedding medium. × 90,000

Fig 13 An organism with fully formed oocyst wall. The cytoplasm contains a nucleus with a distinct nucleolus, number of polysaccharide granules and few remnants of the WFB II (arrows) The irregular shape of the oocyst is believed to be an artifact caused by slight shrinkage because the oocyst wall represents major barrier to fixatives and other reagents. × 7,500

Figures 14–16 are electron micrographs which illustrate the structure of the walls of oocysts obtained from chicken faeces The loose ell seen around oocysts present in the intestine of the host is absent in this material. I these oocysts, the wall consists of an outer layer which could be differentiated into 3 zones and an inner homogeneous layer. Variations are observed in the granular zone of the outer layer of the oocyst wall The material present exterior to the outer oocyst wall is the cross linked BSA used for primary embedding.

Fig 14 The granules form zone of nearly uniform thickness. × 90,000.

Fig 15 The granules are present in aggregates or clusters × 90,000

Fig 16 The granules are absent. × 90,000.

inner zone was more osmiophilic than the first two and only 10–30 nm thick (Figs. 10 & 12)

The inner layer of the oocyst wall was homogeneous in appearance and approx. 80 nm in thickness (Fig 12)

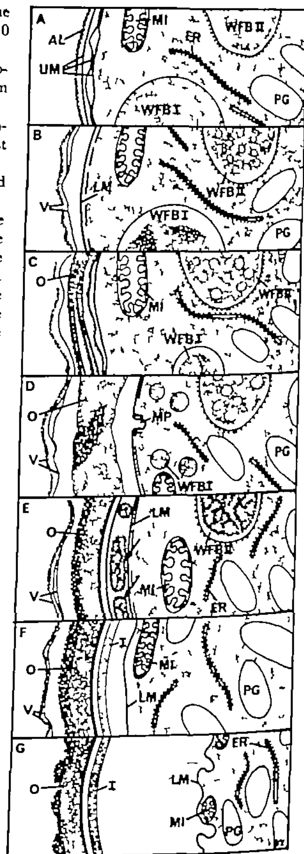
It was impossible to distinguish unit membranes delimiting the two layers of the oocyst wall

The structure of the oocyst wall as observed in oocysts obtained from chicken faeces, differed slightly from that observed in the mature oocyst before excretion from the host cell. The oocysts from the faecal material lacked the delicate membranous veil (Figs. 14 15 & 16) The two layers of the oocyst wall were similar to those observed on oocysts from the intestine (cf Figs 12 & 14) except that the outer granular zone of the outer layer in certain cases, was absent (Fig 16) or reduced in width and in others presented the granules in clusters or aggregates (Fig 15)

A diagrammatical representation of the ultrastructural changes observed during oocyst wall formation is given in Text Fig 1

DISCUSSION

The presence of membranes (in *E brunetti* forming a loose veil) exterior to the two layers of the oocyst wall has previously been reported for a number of *Eimeria spp* although the number of membranes present in the different species varied (2 3 4 11 17 & 20) In *E brunetti* as in the other *Eimeria spp* it appeared that the membranes of the veil are formed by a separation of the limiting membranes of the macrogamete The membranous nature of the veil observed in this study corresponds well to that referred



Text Figure 1 Diagrams A to F summarise the sequential changes observed during the formation of the oocyst wall in *E. brunetti* In Diagram E patches of material similar in appearance to the contents of the WFB II are marked by arrows. Diagram G represents the structure of the wall of oocysts obtained from chicken faeces

to as Layers 1, 2 & 3 of the oocyst wall of *Toxoplasma gondii* (8). In *T. gondii* however, these layers appeared to be synthesized exterior to the limiting membrane.

From the structural changes occurring in the WFB I it would seem that part of their contents are being released prior to the initiation of the formation of the outer layer of the oocyst wall. Changes in the structure of the WFB I observed prior to oocyst wall formation have also been reported for *E. labbeana* (20) *and E. mivati* (22).

The formation of the outer layer of the oocyst wall differed from that reported for other *Eimeria spp.* and *T. gondii* (8 & 14). In *E. brunetti* it appears that this layer is formed from membranes released from the surface of the macrogamete in combination with the contents of the WFB I. In other *Eimeria spp.* it is thought that the WFB I coalesce at the periphery of the organism thus forming the outer layer of the oocyst wall (15 & 14). In *E. necatrix* (4), *E. mivati* (22) and *T. gondii* (8) the formation of this layer is reported as occurring from granular material released by WFB I. The disappearance of the WFB I during the formation of the outer layer of the oocyst wall suggests that these bodies are taking part in this formation.

We found that the outer layer of the oocyst wall condensed into a structure which exhibited three well defined zones. A similar zonal partition of this layer has not been observed for other *Eimeria spp.* or *T. gondii* although some granular material has been reported on the exterior of the outer layer of oocysts of *E. necatrix* when observed in the intestinal lumen (4). In *E. callospermophili* and *E. larimerensis* (12), *Isospora canis* (19) and *I. serra* and *I. anaerna* (18) granular material was also present on the outer wall of oocysts obtained from faecal material.

Between the outer layer of the oocyst wall and the limiting membrane of the cytoplasmic mass of the organism we observed some material with the same density and structure as that present within the WFB II. Similar observations have been reported for *E. acervulina* (10) during the formation of the inner layer of the oocyst wall. Both these observations provide evidence for the involvement of the WFB II in the formation of the inner layer of the oocyst wall.

The finding that the WFB I and WFB II give rise to the outer and inner layers of the oocyst wall, respectively, is similar to that reported for the other *Eimeria spp.* studied (14, 15 & 16).

In other members of the coccidia, which have been studied, belonging to families related to the Eimeriidae, typical WFB I & II are absent and the process of oocyst wall formation is different (for references see *Scholtyseck et al.* 14).

Micropores are present on the limiting membrane of the cytoplasmic mass below the developing oocyst wall in *E. brunetti*. Similar observations have also been reported for *E. tenella* (11), *E. necatrix* (4), *E. labbeana* (21) and *T. gondii* (8), but the functional significance of the presence of micropores during oocyst wall formation is unclear.

The cytoplasmic mass of the oocysts of *E. brunetti* contains large amounts of reserve food material (polysaccharide granules) which is similar to that reported for other *Eimeria spp.* and *T. gondii* (8 & 13). Probably some of these polysaccharide granules will be used during the process of sporulation. Large amounts of rough endoplasmic reticulum have been reported to be present during oocyst formation in *E. ferrisi* (2) but we found no evidence of this in the developing oocyst of *E. brunetti*.

The region between the outer and inner layers of the oocyst wall has unusual properties. In the present study, Vestopal W was used for embedding. However, similar problems were encountered by *Ferguson et al.* (8) when embedding the developing oocysts of *T. gondii* in Araldite. In general, the interior of the oocyst was reasonably well fixed and embedded but the two layers of the oocyst wall seemed to be weakly joined and tended to separate (Figs. 12 & 13), often to such a degree that the interior of the oocyst had fallen out of the sections available for examination. It would appear that, in this particu-

lar region there is present either a repellent substance or an inhibitor of polymerisation which prevents penetration or proper cross linking of the Vestopal or Araldite.

When the ultrastructure of the wall of oocysts obtained from faeces is compared to that of oocysts present within the epithelial cells, only minor differences are observed The loose veil is lost and this is not completely un expected since the oocysts have undergone a number of mechanical and chemical treat ments during the processing of the faeces. Slight differences are observed in the granular zone of the outer layer and these are probably also explained as results of the handling of the faecal material It has been stated that considerable changes occur in the structure of the oocyst wall after excretion with the faeces (18) In the oocysts of *E brunetti* however only slight differences are observed in the walls after excretion and we are thus unable to support this idea.

We are indebted to the *Central Veterinary Laboratory* Ministry of Agriculture Fisheries and Food, New Haw Weybridge Surrey England for supplying a pure sample of oocysts of *E. brunetti* and to *K L. Fennestad* V.M.D., Statens Seruminstitut, for provision and maintenance of the chickens We gratefully acknowledge Mrs. *H Rasn* for technical assistance, and Miss *A Overgaard* and Mr *F Laursen* for photographic assistance

The work was supported by grants from the *World Health Organisation* Geneva *The Wellcome Trust* the *British Council* and the *W H Ross Foundation*

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Acta path microbiol. scand. Sect. B 85 212–218, 1977

THE TENDENCY OF SMOOTH AND ROUGH *SALMONELLA TYPHIMURIUM* BACTERIA AND LIPOPOLYSACCHARIDE TO HYDROPHOBIC AND IONIC INTERACTION, AS STUDIED IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

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Magnusson, K. E., Stendahl, O., Tagesson, C., Edebo, L. & Johansson, G. The tendency of smooth and rough *Salmonella typhimurium* bacteria and lipopolysaccharide to hydrophobic and ionic interaction, as studied in aqueous polymer two-phase systems. Acta path. microbiol. scand. Sect. B 85 212–218, 1977

In aqueous two-phase system, the partition of bacteria and lipopolysaccharide from a rough (R) strain (Rd-mutant) of *Salmonella typhimurium* is influenced by polymers with covalently linked hydrophobic groups indicating hydrophobic structures accessible at the cell surface. Furthermore, the partition of the R bacteria is influenced by a number of inorganic positive and negative ions, presumably as a consequence of interaction with negatively charged surface structures. In contrast, smooth (S) bacteria and lipopolysaccharide from the parent strain do not seem to participate in either hydrophobic or charge interaction indicating extensive hydrophilicity without charge. Thus, the S-specific polysaccharide side chain of *S typhimurium* might serve the purpose of blindfolding aspecific host defence mechanisms dependent on hydrophobicity and charge. On the contrary, the R bacteria and R lipopolysaccharide have physico-chemical properties which predispose to interaction with several types of cells, organelles and molecules.

Key words: *Salmonella typhimurium*; bacteria and lipopolysaccharide; hydrophobic and ionic interaction; aqueous polymer two-phase systems.

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Received 8.xii.76   Accepted 5.i.77

The liability of *Salmonella typhimurium* R bacteria to phagocytosis by polymorphonuclear neutrophils (PMN) (18), clearance from the blood stream to the reticuloendothelial system (21) and attachment to HeLa cells *in vitro* (12) is much greater than that of S bacteria. The lack of S-specific polysaccharide side chains in the surface lipopolysaccharide (LPS) of R bacteria is well known

TABLE 1. *Influence of Deoxycholate-PEG on the Partition of Bacteria, LPS and Alkali-treated LPS (aLPS) in an Aqueous Two-phase System*

Per cent (w/w) deoxycholate-PEG in two-phase system	Per cent in top phase					
	Strain		LPS*		aLPS	
	MS	R10	MS	R10	MS	R10
0	67	3	90	3	95	8
0.004	63	2	92	3	93	10
0.022	66	3	95	5	98	19
0.088	69	3	85	10	96	41

Bacteria and LPS labelled with ^{51}Cr

(15) The loss of the side chains affects the physico-chemical properties of the bacteria, as shown by the close relationship between the partition of the bacteria and their LPS in uncharged and charged polymer two-phase systems (1 22) Except for information about charge (22) conclusions from the partition experiments with respect to other physico-chemical properties determining the difference in partition were indirect. However it has recently been shown that, by the addition of poly(ethylene glycol) (PEG) or dextran with covalently bound hydrophobic side chains to ordinary PEG-dextran two-phase systems, the interaction between the hydrophobic side chains and molecules or particles in the two-phase systems can be investigated. Thus it is assumed that the hydrophobic ligand of the PEG interacts with any hydrophobic structures available. The PEG part tends to accumulate with other PEG molecules, which tends to transfer the whole complex to the PEG-rich phase (17 24 26)

The present investigation aims at further characterization, by means of the two-phase systems, of rough (Rd-mutant) and smooth *S typhimurium* bacteria and LPS with respect to Eability to ionic and hydrophobic interaction.

MATERIALS AND METHODS

Bacteria The smooth, virulent *S typhimurium* 395 MS and its Rd-mutant *S typhimurium* MR10 have been described previously (19) The strains were kept at 4 C on agar slants and their phage patterns checked at intervals. Bacteria were grown overnight (ca 16 h) at 37 C, washed three times and resuspended in phosphate buffered saline (PBS, pH 7.3) For radioactive labelling, ^{51}Cr was used (22)

Lipopolysaccharide (LPS): Bacteria were grown in a liquid synthetic medium with aeration. LPS was extracted from S bacteria with hot phenol-water (23) and from R bacteria with phenol-chloroform-petroleum ether (7) The LPS was labelled with ^{51}Cr similar to *Brande et al.* (3) Briefly the LPS was suspended in distilled water to concentration of 5 mg/ml. Then 7 ml of this suspension was mixed with 0.5 ml $Na_2{}^{51}CrO$ (0.5 mCi) and incubated at 37 C. After 24 h the mixture was dialysed against water until no significant radioactivity was detected in the dialysate. Finally the labelled LPS was concentrated to approximately 10 mg/ml in PBS.

Modified polymers: Palmitoyl-PEG was prepared according to *Shanbhag & Johansson* (17) and contained 0.13 mmol palmitic acid per g polymer Deoxycholic acid was esterified with PEG with the aid of dicyclohexylcarbodiimide (26) and the product contained 0.026 mmol bound acid per gram (deoxycholate-PEG) Palmitoyl-dextran was prepared by washing 10 g dextran (mol.wt. = 500,000) with dry pyridine and after suspension of the polymer in this solvent 2.5 g palmitoyl chloride was added. After 2 h at 40° C the polymer was collected by suction filtration, washed with 100 ml warm (50° C) absolute ethanol and dried at room temperature. The degree of substitution was not determined

Phase systems and partition analysis A two-phase system was prepared from stock solutions of 20 per cent PEG 6000 (Carbowax 6000, Union Carbide, New York, N Y) 20 per cent dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden) 0.1 M tris (hydroxymethyl) aminomethane (tris) buffer pH 7.0 and distilled water It was allowed to equili-

brate at 4 C overnight. The total system contained 4.4 per cent (w/w) PEG and 6.2 per cent (w/w) dextran in 0.03 M tris buffer. A batch of some hundred ml was usually prepared. Two ml of bottom phase and two ml of top phase were pipetted into graded test tubes. To each tube, 0.1 ml bacteria or LPS and 0.1 ml of hydrophobically substituted polymer in tris buffer were added. In order to study the effect of different ions on the partition the tris buffer was exchanged with a 0.5 mM phosphate buffer pH 7.0 (See Fig 5). The tubes were inverted repeatedly (× 20) for mixing and the phases were allowed to settle for 45 min. The volumes of the phases were measured. Then 0.5 ml samples were withdrawn from the top and the bottom phase. The remaining liquid containing the interface was finally mixed and a sample taken. Determination of the bacteria (viable) in the phases was made by diluting each aliquot 1:10 and measuring the optical density at 650 nm in a spectrophotometer (Beckman DU 2 Beckman Instrument Inc., Fullerton, Cal 92634 U.S.A.) or by measuring the radioactivity in a gamma scintillation counter (Intertechnique SA, Paris, France). The latter procedure was also used for the ^{51}Cr LPS. The distribution of the bacteria and LPS between the phases was then assessed on the basis of the volumes of the phases. Due to inhomogeneous distribution in the phases, in certain cases the recovery of particles may exceed 100 per cent (See e.g. Fig 2).

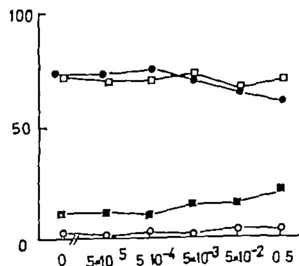


Fig 1 Partition of viable bacteria, *S typhimurium* R10 (open symbols) and MS (filled symbols) in the presence of different concentrations of Triton X 100. ○ = top phase, □ = bottom phase. Ordinate: Per cent bacteria in top and bottom phase. Abscissa: Final concentration of Triton X 100 (per cent w/w)

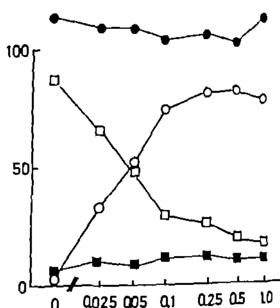


Fig 2 Partition of ^{51}Cr labelled LPS *S typhimurium* R10 (open symbols) and MS (filled symbols) in the presence of different concentrations of Triton X 100. ○ = top phase, □ = bottom phase. Ordinate: Per cent LPS in top and bottom phase. Abscissa: Final concentration of Triton X 100 (per cent w/w)

RESULTS

Influence of deoxycholate PEG. The presence of deoxycholate-PEG in concentrations ranging between 0 and 0.88 per cent (w/w) did not influence the partition of MS and R10 bacteria (Table 1). Neither was the partition of their LPS markedly affected, there being only a slight increase in the partition to the top phase for R LPS. More conspicuous, however, was the accumulation of alkali treated LPS (aLPS preparation see 15) into the top phase promoted by deoxycholate-PEG in the phase system.

Influence of Triton X 100. Triton X 100 in total concentrations 0.00005–0.5 per cent (w/w) in the phase system had little effect on the partition of the bacteria. The R10 was hardly affected, whereas the MS bacteria were decreased slightly in the top phase from 74 to 60 per cent and moved toward the interface and bottom phase (Fig 1). This might be due to damage of the cell envelope in the high concentration of detergent. With R10 LPS, increasing concentrations of Triton X 100 promoted the accumulation into the top

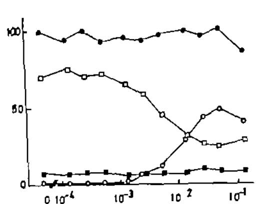


Fig 3 Partition of viable bacteria, *S typhimurium* R10 (open symbols) and MS (filled symbols) in the presence of different concentrations of palmitoyl-PEG ○ = top phase, □ = bottom phase.
Ordinate Per cent bacteria in top and bottom phase
Abscissa. Final concentration of palmitoyl-PEG (per cent w/w)

phase. In the absence of Triton X 100 85 per cent of the LPS was recovered from the bottom phase as compared to only 20 per cent with 1 per cent (w/w) Triton in the phase system The partition of the MS LPS was not affected by Triton (Fig 2)

Influence of palmitoyl-PEG and palmitoyl-dextran. The presence of palmitoyl-PEG promoted the partition of R10 bacteria to the top phase at final concentrations of 0.002 per cent (w/w) and greater (i.e. about 0.05 per cent of the PEG exchanged with PEG with hydrophobic groups) and higher whereas there was no effect on the partition of MS (Fig 3) Only the highest concentration (10 per cent) gave a small decrease in the top phase. No conclusive effect of palmitoyl dextran in concentrations 0–0.25 per cent (w/w) was observed, either on R10 or MS bacteria (not shown by figure)

With R10 LPS palmitoyl-PEG in concentrations of 0.001–0.002 (w/w) and greater increased the affinity for the PEG-rich top phase whereas the MS LPS was not affected (Fig 4) Palmitoyl-dextran at 0.25 per cent (w/w) and lower did not influence either the distribution of R10 LPS or of MS LPS (not shown by figure)

Partition of bacteria in the presence of inorganic salts. The effect of 0.025 M concentrations of potassium halides or alkali chlorides on the partition of MS (^{51}Cr labelled) was small or negligible whereas the partition of R10 (^{51}Cr labelled) depended on the particular ion (Fig. 5) Li and F had little or no effect, whereas the other ions promoted the accumulation into the bottom phase. Arranged in increasing order of anions F <Cl <Br <I and of cations Li <Na <K (Fig. 5) It is notable that in comparison with the tris buffer the phosphate buffer used in this context moves the R10 bacteria from the bottom phase towards the interface.

DISCUSSION

The attachment of *S typhimurium* R bacteria to different kinds of animal cells such as granulocytes (18) macrophages (6) HeLa cells (12) and amoeba (8) is more avid than that of S bacteria. Since the difference is evident also in the absence of serum, aspecific attachment mechanisms, such as charge and hydrophobicity have been postulated (19) In line with this hypothesis, physico-chemical

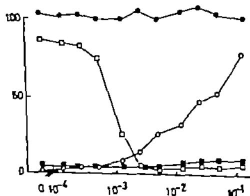


Fig 4 Partition of ^{51}Cr-labelled LPS, *S typhimurium* R10 (open symbols) and MS (filled symbols) in the presence of different concentrations of palmitoyl-PEG ○ = top phase, □ = bottom phase.
Ordinate Per cent LPS in top and bottom phase.
Abscissa. Final concentration of palmitoyl-PEG (per cent w/w)

brate at 4 C overnight. The total system contained 4.4 per cent (w/w) PEG and 6.2 per cent (w/w) dextran in 0.05 M tris buffer. A batch of some hundred ml was usually prepared. Two ml of bottom phase and two ml of top phase were pipetted into graded test tubes. To each tube, 0.1 ml bacteria or LPS and 0.1 ml of hydrophobically substituted polymer in tris buffer were added. In order to study the effect of different ions on the partition, the tris buffer was exchanged with a 0.5 mM phosphate buffer pH 7.0 (See Fig. 5). The tubes were inverted repeatedly (× 20) for mixing and the phases were allowed to settle for 45 min. The volumes of the phases were measured. Then 0.5 ml samples were withdrawn from the top and the bottom phase. The remaining liquid containing the interface was finally mixed and a sample taken. Determination of the bacteria (viable) in the phases was made by diluting each aliquot 1:10 and measuring the optical density at 650 nm in a spectrophotometer (Beckman DU 2, Beckman Instrument Inc., Fullerton, Cal. 92634, U.S.A.) or by measuring the radioactivity in a gamma scintillation counter (Intertechnique SA, Paris, France). The latter procedure was also used for the [51]Cr-LPS. The distribution of the bacteria and LPS between the phases was then assessed on the basis of the volumes of the phases. Due to inhomogeneous distribution in the phases, in certain cases the recovery of particles may exceed 100 per cent (See e.g. Fig. 2).

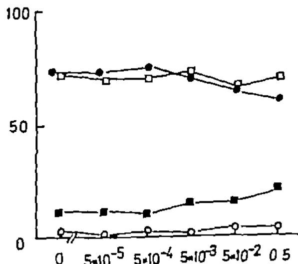


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Abscissa: Final concentration of Triton X 100 (per cent w/w).

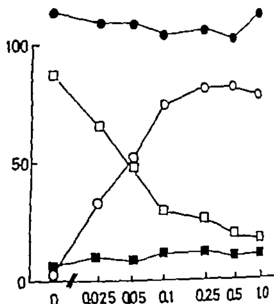


Fig. 2. Partition of [51]Cr-labelled LPS *S. typhimurium* R10 (open symbols) and MS (filled symbols) in the presence of different concentrations of Triton X 100. ○ = top phase, □ = bottom phase. Ordinate: Per cent LPS in top and bottom phase. Abscissa: Final concentration of Triton X 100 (per cent w/w).

RESULTS

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S bacteria did not react with the different hydrophobic groups either they seem to be provided with surface properties capable of blindfolding and escaping aspecific host defence mechanisms which operate via surface hydrophobicity or charge Hydrophilic properties of S bacteria have long been considered responsible for their more stable suspensions, virulence and resistance towards phagocytosis (5) and this is now being substantiated.

On the contrary R bacteria and LPS showed greater tendency to interaction with hydrophobic and charged groups. Already in 1935 *Mudd et al.* (14) postulated on the basis of theoretical considerations, that phagocytes would more easily become attached to hydrophobic particles, and in the words of *van Oss* (23) phagocytes do not distinguish between self and nonself but just between particles that are more hydrophilic or more hydrophobic than themselves" Besides the importance of the physico-chemical characteristics in relation to pathogenic mechanisms, the R bacteria and LPS are more reactive in a variety of biological interactions. They are more rapidly cleared from the blood stream (10) and more toxic to mitochondria (9) Furthermore, the lipid A linked mitogenicity on bone marrow derived lymphocytes is more pronounced with LPS from *S typhimurium* SL 684 grown in the absence of galactose (Rc type LPS) than in the presence of galactose (S-type LPS) (2, 16)

To summarize there seems to be a general tendency of the R-type of enterobacteria and their LPS to interact with mammalian cell membranes to a greater extent than S bacteria The lower effect in the presence of S-specific polysaccharide (PS) chains may be visualized in two different ways 1 The PS side chains provide a steric hindrance to the specific interaction of the lipid A portion with receptors in the mammalian cell membrane 2 The PS side chains increase the hydrophilicity of the particles, so that short range aspecific interactions through, for instance van der Waal's bonds and hydrophobic interaction with the animal cell membranes, are impaired. Since hydrophobicity seems to facilitate the interaction and bonding we favour the second type of interpretation as the basic mechanism. Whether this relationship is representative of all *Salmonella* S and R strains remains to be proved experimentally Such a mechanism would, however have general importance in the interaction of animal cell membranes with extra cellular material.

The higher virulence of S bacteria of *S typhimurium* than that of R bacteria is thus not due to higher toxicity or reactivity towards the host cells, but is a consequence of escape mechanisms which allow the bacteria to slip through the imperfect walls of the mucous membranes and evade the aspecific defence mechanisms of phagocytes.

The skilled technical assistance of *Kerstin Hagerstam* is gratefully acknowledged. This research was supported by the grant B 76-16 X-02103-10 from the *Swedish Medical Research Council.*

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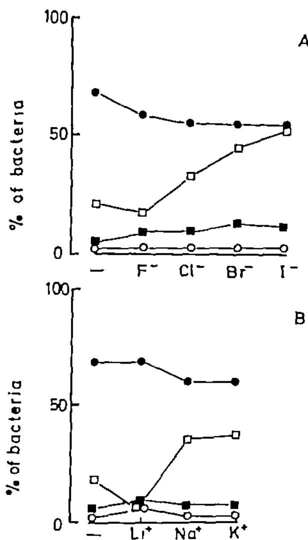


Fig 5 Partition of viable bacteria, *S typhimurium* R10 (open symbols) and MS (filled symbols) in a dextran polyethylene glycol two-phase system containing 25 mM sodium halides (A) or alkali chlorides (B) Phase composition 6 2 per cent (w/w) dextran, 4 4 per cent (w/w) PEG 0 5 mM phosphate buffer pH 7 0 25 mM chlorides or halides. ○ = top phase, □ = bottom phase.

differences between the two kinds of bacteria have been demonstrated by partition in polymer two-phase systems consisting of dextran and PEG (19 20) Recently it was shown by means of a phase system with charged PEG that R10 has a net negative charge whereas MS is apparently uncharged (22) However no conclusion with respect to hydrophobic mechanisms was justifiable on the basis of those experiments. Partition in the two-phase system has been found to correlate with contact angle measurements since the presence of *S typhimurium* S type LPS on the bacterial surface reduces the contact angle in consequence of hydrophilic effects (4) Thus, in the two-phase system, virtually uncharged and hydrophilic S bacteria prefer the PEG-rich top phase and less hydrophilic and negatively charged R bacteria prefer the dextran rich bottom phase. More direct proof of the hydrophobicity of R bacteria comes from the present experiments, since PEG and dextran covalently linked with palmitoyl side chains especially palmitoyl PEG were observed to influence the partition of R bacteria and LPS but not that of S bacteria (Figs 1–4)

The conspicuous effect of palmitoyl-PEG on the partition of R10 in contrast to deoxycholate-PEG or Triton X 100 (octylphenyl-PEG) might indicate that steric properties of the hydrophobic moiety played a role so that interaction was favoured with less bulky chain-like structures. A similar effect was observed with R10 LPS where a much smaller concentration of palmitoyl-PEG (Fig 4) than that of deoxycholate PEG (Table 1) or of Triton X 100 (Fig 2) was needed to affect the partition The capacity of deoxycholate-PEG to affect the partition of R10 LPS and still more of R10 aLPS (Table 1) indicates that the hydrophobic moieties were more accessible in these preparations, or that they quantitatively dominated the surface properties of the particle to a larger extent (see 13)

The addition of alkali halides to a dextran-PEG system probably leads to an interaction between PEG and the negative halide ions, so that it behaves as negatively charged (11) The order of the effect of the halides (F < Cl < Br < I) on the partition of R10 (Fig 5) corresponds to their degree of interaction with PEG and the effect on the partition of ovalbumin at pH 6.9 (11) Also the effect of cations on the partition of R10 was similar to that on ovalbumin (11) These results are consistent with the negative surface charge of R10 (22) The very small effect of ions on S bacteria and LPS was conspicuous, thus indicating negligible charge interaction. Since

INHIBITION OF THE SYNTHESIS OF PHOSPHOLIPASE C IN *BACILLUS CEREUS* BY A COMPONENT OF THE GROWTH MEDIUM

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Valle, K. J Otnæss A. B. & Prydz, H. Inhibition of the synthesis of phospholipase C in *Bacillus cereus* by a component of the growth medium. Acta path. microbiol. scand. Sect. B, 85 219-226 1977

A low molecular weight compound, probably of peptide nature present in the Beef Heart Extract component of the growth medium, inhibits post-transcriptionally the biosynthesis of phospholipase C by a strain of *Bacillus cereus*. The compound also prevents the increase of proteolytic activity in the growth medium, thus suggesting that the synthesis of another enzyme an extracellular protease, is also inhibited, and that the inhibitory compound may therefore have more general effect on exoenzyme synthesis in this strain of *Bacillus cereus*.

Key words Phospholipase C inhibitor of exoenzymes; *Bacillus cereus* exoenzyme synthesis.

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Received 9.xii.76 Accepted 15.i.77

Phospholipase C (EC 3.1.4.3.) (PLC) is an exoenzyme produced by some members of the family *Bacillaceae*. In recent years PLC from *Bacillus cereus* has been the subject of a number of investigations in our (2, 13 15 16, 19-24) and in other laboratories (12, 25 30, 31) We have previously reported that a low molecular weight compound present in the Beef Heart Extract-component of the Brain Heart Infusion medium (Oxoid) completely inhibited the synthesis of PLC without interfering with bacterial growth or sporulation (23) Further studies on this compound and its mode of action are reported here.

MATERIAL AND METHODS

Bacterial Strain and Cultivation

Bacillus cereus ATCC 10987 was stored at 4 C on nutrient agar and grown in liquid culture as described (23) using nutrient broth, Brain Heart Infusion (Oxoid) or their low molecular weight fractions obtained by dialysis of growth media. In some experiments the medium described by *Zwaal et al.* (30) or a 1 per cent maltose-0.5 per cent casein hydrolysate medium (Difco) (8) was used. In other cases 1 per cent glucose and 0.5 per cent unhydrolysed casein (Merck) were used instead of maltose and casein hydrolysate. Growth was monitored at 600 nm.

Enzyme Assays

Phospholipase C was assayed as described by *Otnæss et al.* (24) One PLC unit inactivated one unit of a standard preparation of tissue thromboplastin per min at 37 C. Proteolytic activity was determined as described by *May & Elliott* (18)

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INHIBITION OF THE SYNTHESIS OF PHOSPHOLIPASE C IN *BACILLUS CEREUS* BY A COMPONENT OF THE GROWTH MEDIUM

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Valle, K. J., Otnæss, A. B. & Prydz, H. Inhibition of the synthesis of phospholipase C in *Bacillus cereus* by a component of the growth medium. Acta path. microbiol. scand. Sect. B 85: 219–226, 1977

A low molecular weight compound, probably of peptide nature, present in the Beef Heart Extract component of the growth medium, inhibits post-transcriptionally the biosynthesis of phospholipase C by a strain of *Bacillus cereus*. The compound also prevents the increase of proteolytic activity in the growth medium, thus suggesting that the synthesis of another enzyme, an extracellular protease, is also inhibited, and that the inhibitory compound may therefore have a more general effect on exoenzyme synthesis in this strain of *Bacillus cereus*.

Key words: Phospholipase C; inhibitor of exoenzymes; *Bacillus cereus*; exoenzyme synthesis.

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Received 9.xii.76 Accepted 15.i.77

Phospholipase C (EC 3.1.4.3) (PLC) is an exoenzyme produced by some members of the family *Bacillaceae*. In recent years PLC from *Bacillus cereus* has been the subject of a number of investigations in our (2, 13, 15, 16, 19–24) and in other laboratories (12, 25, 30, 31). We have previously reported that a low molecular weight compound present in the Beef Heart Extract-component of the Brain Heart Infusion medium (Oxoid) completely inhibited the synthesis of PLC without interfering with bacterial growth or sporulation (23). Further studies on this compound and its mode of action are reported here.

MATERIAL AND METHODS

Bacterial Strain and Cultivation

Bacillus cereus ATCC 10987 was stored at 4° C on nutrient agar and grown in liquid culture as described (23) using nutrient broth, Brain Heart Infusion (Oxoid) or their low molecular weight fractions obtained by dialysis of growth media. In some experiments the medium described by *Zwaal et al.* (30) or a 1 per cent maltose-0.5 per cent casein hydrolysate medium (Difco) (8) was used. In other cases 1 per cent glucose and 0.5 per cent unhydrolyzed casein (Merck) were used instead of maltose and casein hydrolysate. Growth was monitored at 600 nm.

Enzyme Assays

Phospholipase C was assayed as described by *Otnæss et al.* (24). One PLC unit inactivated one unit of a standard preparation of tissue thromboplastin per min at 37° C. Proteolytic activity was determined as described by *May & Elliott* (18)

using casein or azo-casein (Sigma) as substrate. Amylase was determined as described by *Coleman & Elliott* (9)

Gel Filtration

Bio-Gel P 10 and P 2 (Bio-Rad) were used in columns of 2.8 × 61 cm and 2 8 × 50 cm. Standard procedures were followed The columns were equilibrated and eluted with 5 mM Na phosphate buffer pH 7 4 in 0 15 M NaCl at a rate of 20 ml/h.

Antisera

Antisera against PLC were raised as described (*Otnaess et al. 1976* in preparation) by injection of purifed (15) enzyme into goats. The monospecific antibodies obtained were isolated as described by *Fahey* (11) and further purifed by using a PLC-Sepharose affinity column (*Otnaess et al*, 1976 in preparation)

Thin-layer Chromatography

Thin layer chromatography (TLC) was carried out on 0.3 mm silica gel H (Merck) using n-butanol acetic acid water (80 20 20) as solvent and ninhydrin (Koch-Light) for detection.

Paper Chromatography

One dimensional chromatography on Whatman No 1 paper was used. The solvents were n-butanol acetic acid water (40 6 15) and isopropanol formic acid water (70 20 10) The spots were detected with ninhydrin.

Amino Acid Analyses

Amino acid analyses were carried out on a Jeol JLC-6AH analyzer after hydrolysis of the inhibitor fraction in 6 N HCl for 18h at 110 C.

Scanning Electron Microscopy (SEM)

The cells were fixed for 2 h in 2.5 per cent glutaraldehyde in 0 08M sucrose, dehydrated with increasing ethanol concentrations and transferred to pentyl acetate. Finally the cell suspensions were placed on glass cover slips and subjected to critical point drying with CO_2. The specimens were coated with carbon and gold by vacuum evaporation and examined in a Hitachi (HHS/2R) scanning electron microscope at 20 kV and a 30 tilt angle.

Isotope Experiments

200 µl L-(U-¹ C)-leucine (specific activity 348 Ci/mol, 50 µCi/ml) and 12 µl 5 6-³H uridine (specific activity 41 Ci/mmol, 1 mCi/ml) (Radiochemical Centre, Amersham) were added to 15 ml medium. The bacterial cell pellets were extracted with 2 ml ice cold 0.5 M perchloric acid (PCA) for 15 min at 2 C, centrifuged (10,000 g for 10 min at 4 C) and washed with ice cold PCA five times. The last PCA-extract was counted and found to be free from significant radioactivity The final pellet was dissolved in 1 ml 0.5 M NaOH for 48 h at 37 C in closed tubes. From each hydrolysate three samples (0.1 ml) were counted in a Packard Tricarb 3375 Scintillation Counter after mixing with 10 ml Instagel. Quenching was found to be insignificant.

Other Methods

Protein was determined by the Lowry method (17) using bovine serum albumin as standard. Hexoses were determined by the phenol-sulphuric acid method of *Briggs et al.* (5) pentoses by the orcinol method (27) deoxyribose by Burton's method (6) phosphorus as described by *Chen et al.* (7) and arginine by a spot test according to *Stahl* (26) Free amino groups were determined by the ninhydrin reaction at 570 nm. Solutions of Pronase (Calbiochem) (1 mg/ml) were autodigested for 1 h at 37 C before use.

RESULTS

The low molecular weight fraction of Beef Heart Extract completely inhibited the appearance of PLC in the growth medium of certain substrains of *B cereus* ATCC 10987 (Fig 1) In confirmation of earlier results (23) no in-

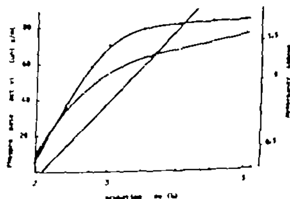


Fig 1 Effect of low molecular weight fraction of Brain Heart Infusion on phospholipase C activity and bacterial growth in liquid culture.
—○— Absorbancy at 600 nm and —△— phospholipase C activity for cells growing in the low molecular fraction of nutrient broth.
—●— Absorbancy at 600 nm and —▲— phospholipase C activity for cells growing in the low molecular fraction of Brain Heart Infusion.

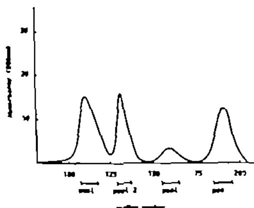


Fig. 2 Gel filtration of partially purified inhibitor on Bio-Gel P 2. The active fractions from a Bio-Gel P 10 column were pooled, freeze-dried, dissolved in distilled water and rechromatographed on a P-2 column (2.8 × 50 cm) equilibrated and eluted with phosphate-buffered saline. Fractions of 2.2 ml were collected at rate of 20 ml/h.

crease of intracellular PLC activity was found when bacteria inhibited by the Beef Heart Extract were broken by homogenization, alumina grinding or sonication. No antigenic material was found when the monospecific antibodies against PLC were used in immunodiffusion against homogenates or normal or inhibited bacteria (5 × 10⁹ cells/ml)

Characteristics of Inhibitor Preparations

A partial purification of the inhibitor was obtained by successive gel filtrations on Bio-Gel P 10 and P 2 columns (Fig 2) The final preparations (Pool 3) completely inhibited the increase in PLC activity (Table 1) Several tests were carried out to gain information about the nature of the inhibitor and its mode of action. Calibration of the P 2 column with vitamin B_{12}, riboflavin and tyrosine suggested a molecular weight for the inhibitor of 600-900 provided significant adsorption of the inhibitor to the gel matrix did not take place. The inhibitor was heat stable and active after autoclaving at 120 C for 15 min in growth medium. Digestion of partially purified inhibitor preparation with pronase (final concentration 100 µg/ml at 37 C for 1-2 h) reduced the inhibitory effect by 70-80 per cent. Pronase had no effect on the growth of bacteria in the concentrations used. Protein determination showed that the partially purified inhibitor fraction contained 1.2 mg/ml and completely inhibited the PLC synthesis at 0 6 mg/ml of growth medium. The growth of bacteria was not affected. Significant amounts of hexoses or deoxyribose were not found. The orcinol tests suggested the presence of a very small amount of ribose (3.2 µg/ml) Trace amounts of organic phosphate (1 µg/ml) were detected. Arginine was not found (i.e. less than 1 µg/ml) Ninhydrin tests showed the presence of free amino groups (about 5 per molecule of mol. wt. 6-900)

The ultraviolet spectrum of the preparations had a maximum at 274 nm at neutral pH. The ratio $A_{294\,nm}/A_{274\,nm}$ was 3.38 These data are consistent with the presence of tyrosine but not tryptophan in the preparations. The ratio $A_{260\,nm}/A_{274\,nm}$ was 1.29 thus suggesting that very little or no nucleic acids,

TABLE 1 *Effect of Inhibitor on Growth† and Extracellular Phospholipase C Activity*

Pool	165 min		240 min		330 min		420 min	
	A 600	Units/ml	A 600	Units/ml	A 600	Units/ml	A 600	Units/ml
1	1 15	25	1.80	19	1.85	8	1.90	15
2	0 87	20	1.50	21	1.65	19	1 70	20
3	1 15	0	1.80	0	1 85	0	1.90	0
4	1.37		1.65	22	1 70	13	1 70	6

Pooled fractions as indicated in Fig. 2.
† Absorbancy at 600 nm.

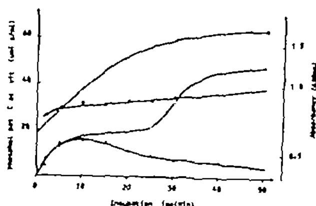


Fig 3 Phospholipase C activity and bacterial growth after resuspension of washed cells.
—○— Absorbancy at 600 nm and —△— phospholipase C activity for cells resuspended in nutrient broth.
—●— Absorbancy at 600 nm and —▲— phospholipase C activity for cells resuspended in nutrient broth containing 60 µg rifampicin/ml

nucleotides or purine and pyrimidine bases were present The UV spectra at pH 2.3 and 7 4 coincided whereas the spectrum at pH 10 7 showed a marked increase at 235-255 nm but no marked change at 274 nm or above. Tyrosine in the preparations is thus not ionized at pH 10 7 Amino acid analyses showed that the preparation contained tyrosine gly cine serine glutamic acid alanine, aspartic acid and threonine in molar ratio 10.5.5.3 9 2.3 1 8 1 7 1 No attempts was made to determine how much glutamic and aspartic acid were derived from glutamine and asparagine

Paper chromatography in the n-butanol system and TLC gave one ninhydrin positive spot. The isopropanol system gave two ninhydrin positive spots, thus showing that the preparation contained at least two different peptides. No ninhydrin positive spots with mobilities corresponding to those of tyrosine glycine or serine were seen

Mode of action of inhibitor

In order to investigate the mode of action of the inhibitor we studied the synthesis of PLC in bacteria harvested when the rate of enzyme synthesis was close to or at its highest level. Such cells from 20 ml culture were harvested by rapid filtration on 0.22 µ Millipore filters, washed with 10 ml fresh medium and resuspended in fresh nutrient broth within 2–3 min The procedure was carried out at 37° C with prewarmed medium. The effect of inhibitors of DNA replication (nalidixic acid (Sigma)) transcription (rifampicin (Lepetit)) and translation (chloramphenicol (Sigma)) was compared with the effect of the unknown inhibitor by adding the respective compounds to the growth medium used for resuspension of washed bacteria.

Resuspension of the cells in nutrient broth without any addition was followed by a biphasic increase in the PLC activity of the medium (Fig 3) as also seen for the extra cellular protease in *Bacillus amyloliquefaciens* (3 4) The first phase lasted 10–20 min and was not inhibited by rifampicin at a concentration wich reduced ^{3}H uridine incorporation by 85 per cent after 5 min and by 91 per cent after 10 min. However the second phase of increased PLC activity was completely abolished.

Nalidixid acid had a similar effect, permitting the first and inhibiting the second phase of increased PLC activity Complete inhibition of DNA replication was obtained with 80 µg/ml, and this concentration was used.

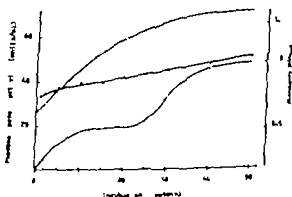


Fig 4 Phospholipase C activity and bacterial growth after resuspension of washed cells.
—○— Absorbancy at 600 nm and —△— phospholipase C activity for cells resuspended in nutrient broth.
—●— Absorbancy at 600 nm and —▲— phospholipase C activity for cells resuspended in nutrient broth containing 400 µg chloramphenicol/ml.

TABLE 2. *Effect of Inhibitor on Growth and Extracellular Proteolytic Activity* †

	75 min		90 min		105 min		120 min		145 min	
	A 600	Units/ml	A 600	Units/ml	A 600	Units/ml	A 600	Units/ml	A 600	Units/ml
Casein medium	0.57	0	0.81	0.1	1.14	0.4	1.45	1.1	1.77	3.0
Casein medium + Inhibitor	0.53	0	0.78	0.1	1.13	0.2	1.47	0.1	1.86	0.2
Casein medium + Casein hydrolysate	0.54	0	0.80	0.1	1.13	0.2	1.45	0.6	1.90	2.2

Absorbancy at 600 nm.
† One proteolytic unit corresponds to an absorbancy increase at 440 nm of 0.5 in 40 min at 37 C with azo-casein as substrate.

Chloramphenicol completely abolished the first as well as the second phase of PLC increase (Fig. 4).

To study the effect of the inhibitor on other extracellular enzymes, we determined the activity of α-amylase and protease(s) in the culture supernatants. Only very low α-amylase activity was detectable. However the inhibitor had a marked effect on the increase of proteolytic activity in the culture supernatants (Table 2). This was not due solely to repression of protease synthesis caused by the addition of amino acids or small peptides with the inhibitor preparation, since the addition of 0.5 casein hydrolysate allowed at least 55-75 per cent of the protease activity to appear in the supernatant (Table 2). Furthermore, the pronase-digested inhibitor was less effective than the undigested inhibitor in inhibiting the increase of proteolytic activity in the culture supernatant. During this work we observed repeatedly that substrains of *B. cereus* ATCC 10987 *previously inhibitable* with respect to PLC synthesis, became uninhibitable. This instability suggested that episomal gene(s) might be involved. In a SEM study of inhibitable and non-inhibitable variants of the same *substrain*, a clear difference was noted. The inhibitor-insensitive cells had a large number of pili-like structures (Fig. 5A) whereas the inhibitor-sensitive cells had no or very few such structures and tended to grow in long chains (Fig. 5B).

DISCUSSION

The inhibitor studied here had no effect on the activity of PLC *in vitro* (23) and did not lead to any detectable intracellular accumulation of PLC activity or antigenically cross-reacting material. Hence, it is probably a true inhibitor of PLC synthesis. This is further confirmed by its immediate effect on the increase of PLC activity which is similar to the effect *of chloramphenicol, thus suggesting that the* inhibition is posttranscriptional.

The nature of the inhibitor remains unknown. Analyses of the partially purified preparations suggest that it is a peptide and make alternatives like carbohydrates, phospholipids, nucleic acids or their components less likely. The *effect of* pronase supports this hypothesis. The products of the PLC reaction have been tested earlier and found to be without effect (23).

The presence of free amino groups (about 5 per molecule of mol. wt. 6-900) is difficult to reconcile with the structure of an ordinary peptide of this molecular weight and amino acid composition. Free amino acids may be present, but this seems unlikely since the preparations were submitted repeatedly to gel filtration and no spots corresponding to free tyrosine, glycine or serine were seen in TLC. The presence of so many free amino groups per molecule therefore remains unexplained at present.

The inhibitor was also effective against the

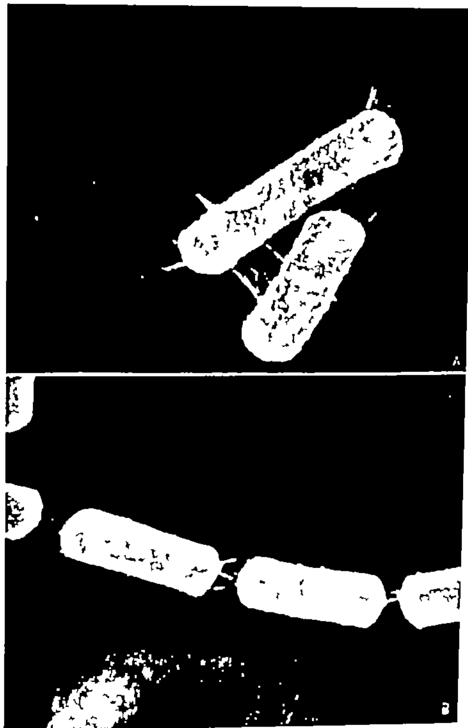


Fig 5
Scanning electron micrographs of *Bacillus cereus* grown in nutrient broth.
A Inhibitor insensitive cells
B Inhibitor-sensitive cells
Primary magnification 10000 ×

increase of proteolytic activity in the culture supernatant. Various extracellular proteolytic enzymes are produced by members of the family *Bacillaceae* (9 10 14) and no attempt was made to characterize further the proteolytic activity studied here.

The possible existence of a peptide which inhibits the synthesis of at least two extracellular enzymes in *B cereus* is interesting in relation to the reports by *Yoneda* and *Maruo* and their collaborators (1 28 29) which suggest a common genetic regulation at some step of exoenzyme synthesis in *Bacillus subtilis*. Our observation of an inhibitor active against at least two different exoenzymes supports the existence of a common step and the results

suggest that the inhibited step is posttranscriptional.

The pleiotropic phenomena reported here in the inhibitor-sensitive and inhibitor insensitive strains otherwise thought to be isogenic, are also very similar to observations by Ayusawa et al. (1) in *B. subtilis*.

Since the extracellular enzymes and toxins are important for the pathogenetic effects of the *Bacillaceae* elucidation of the mechanism of action of this inhibitor might be of practical importance.

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METHODOLOGICAL ASPECTS OF AN AGAR PLATE TECHNIQUE FOR DETERMINATION OF BIOLOGICALLY ACTIVE 5-FLUOROURACIL IN BLOOD URINE AND BILE

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Brandberg, A., Almersjö O Falsen, E Gustavsson, B., Hafström, Lo & Lindblom, G B. Methodological aspects of an agar plate technique for determination of biologically active 5-fluorouracil in blood, urine and bile Acta path microbiol. scand Sect. B, 85 227 234 1977

A microbiological agar plate technique for estimation of 5-fluorouracil concentrations in blood, urine and bile from man, dog and pig was evaluated Different bacterial test strains, media modifications and techniques for inoculation were studied. The strain *Streptococcus faecalis* ATCC 8043 recommended previously by Clarkson et al was found to be the most suitable. The influence of prediffusion, dilution, antibiotics and chemotherapeutic agents and their antagonists, as well as the effect of storage of samples containing 5-fluorouracil were examined A detailed methodological description is presented. The method seems to be sufficiently sensitive and practical for routine determination of cytotoxic compounds from 5-fluorouracil in serum, plasma and urine

Key words 5-fluorouracil microbiological assay agar plate technique.

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Received 3 77 Accepted 2.n 77

The proliferation of malignant cells can be inhibited by cytostatic drugs. 5-fluorouracil (5-FU) seems to be the most effective drug for treatment of metastases from adenocarcinoma of the breast and the gastrointestinal tract (2) The tumour inhibitory properties of 5-FU were originally demonstrated by *Heidelberger & co-workers* in 1957 (7) The chemotherapeutic action of 5-FU is related to the corresponding nucleotide (FUdRMP) which blocks thymidylate synthetase essential for DNA synthesis and cell replication (12) This means that all rapidly growing tissues are influenced by the drug (10) This side effect has restricted the use of the drug in cancer therapy and often constitutes the limitation of the treatment.

Metabolic degradation of 5-FU seems to occur especially in the liver (4) but elimination also takes place through the kidneys (5) Thus, side-effects and the so-called clinical

tumour response to 5-FU treatment vary very much in different individuals in relation to their liver and kidney function

The difficulties in predicting the effects of a given dose of 5-FU necessitate the development of a method for determining the concentration of active drug in tissue and body fluids. Furthermore, such a method is necessary for evaluation of the rationale of different forms of administration of the drug

A microbiological method for estimation of the concentration of 5-FU was described by *Clarkson et al* (1964) This was claimed to be superior to the spectrophotometric methods available because it determines lower levels of the drug and registers only the biologically active substance. The method is based on the fact that the growth of an enterococcus strain (ATCC 8043–*S faecalis*) is inhibited by 5-FU

The aim of the present work was to report the technical problems associated with microbiological determination of 5 FU in blood urine and bile

MATERIAL AND METHODS

The experiences are based on analysis of 3 764 samples of serum, plasma, urine and bile (1 881 from patients 1 169 from dogs and 624 from pigs given 5-FU) Commercial 5-FU (fluorouracil Roche) 50 mg/ml, was used. The enterococcus strain ATCC 8043 (*S faecalis*) was used as reference test organism. A total of 34 different bacterial strains were tested

Capps, Hobbs & Fox s Folic Acid Assay Medium was used for determination of 5-FU This was made uracil free and folic acid was added (Table 1) Different types of standard non-selective bacteriological media were also tested. For sensitivity tests a peptonefree agar with 5 per cent horse blood (3) was used Inoculation of the medium with a test strain was performed either by surface seeding or by the pour plate technique Colonies from an overnight growth were harvested with a 1-microlitre platinum loop suspended in 5 ml nutrient broth and incubated at 37 C for 3 hours For surface inoculation, 3 ml of this culture in 50 ml sterile distilled water was flooded over the plates and the surplus was drained off carefully The plates were dried at 37 C 90 minutes before and 60 minutes after inoculation Using the pour plate technique 30 ml of nutrient broth was inoculated with the bacteria from one colony After incuba-

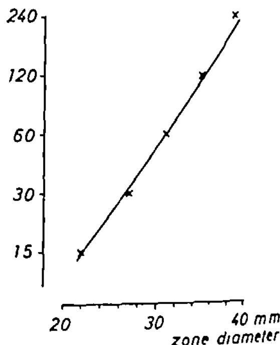


Fig 1 Representative standard curve for 5-FU in human serum.

tion for 4 hours at 37 C, the culture was spun down (4 000 rev/min) and the sediment suspended in 10 ml saline. 2 ml of this suspension was mixed thoroughly with 500 ml of medium. Standard plastic Petri dishes (Nunc, Roskilde, Denmark) with diameters of 9 and 14 cm, were filled with 25 and 55 g of the medium, respectively on a balance and left to solidify on a carefully levelled table.

Three different methods for application of samples were investigated 1) 100 µl of the samples was applied with 50 µl micropipettes into basins (10 mm diameter) cut in the agar plate. The bottom of the basins consisted either of the plastic dish or of agar medium 2) 50 µl of the samples was added to filter paper discs* 10 mm in diameter 3) 10 µl of the samples was added with microcapillar tubes to paper discs 5 mm in diameter The discs were placed on the agar surface with a needle either directly or after being dried. The plates were either incubated immediately or kept at room temperature for 30 or 120 minutes prior to incubation, to allow evaluation of the influence of prediffusion on the zone of inhibition.

The interaction of some generally used antibiotics and chemotherapeutic drugs on the test system for 5-FU with the enterococcus ATCC 8043 was also investigated using the agar diffusion paper disc method (AB Biodisk, Stockholm) (6)

*(AB Biodisk, Solna, Sweden)

TABLE 1 U 348 Modification of the Assay Medium of C pps et al.

		Final concentration (g/l)
Solution A		
d-Biotin, (e.g. Sigma B-4501)	8 mg	(0.0000008 g)
Folic acid	30 mg	(0 000003 g)
Distilled water (g. Millipore or redistilled)	1000 ml	
Solution B		
p-Aminobenzoic acid	20 mg	(0.0002 g)
Calcium-D-pantothenate (Merck)	40 mg	(0.0004 g)
Distilled water (g Millipore or redistilled)	100 mg	
Complete medium		
Vitamin-free Casamino Acids, Difco 0288	12 g	
Glucose A.R. quality	40 g	
Tri-Sodium Citrate $2H_2O$ A.R.	20 g	
L-Cystine A R. or CHR. (BDH or Fluka)	0.2 g	
L-Tryptofan, A.R. (g Merck 8374)	0.1 g	
Adenine sulphate (e.g. Sigma)	0.02 g	
Guanine hydrochloride (g NBCo)	0.02 g	
Thiamine hydrochloride (Vitamin B g. Merck)	0.002 g	
Pyridoxine hydrochloride (Vitamin B_6 g Sigma, P9755)	0 004 g	
Riboflavine (Vitamin B) pure	0 002 g	
Niacine (Nicotinic acid) purum	0 002 g	
D Potassium monohydrogen phosphate, K_2HPO A.R	1 g	
Mono-Potassium dihydrogen phosphate, KH_2PO A.R.	1 g	
Magnesiumsulphate $MgSO_4$. $7H_2O$ A.R	0.4	
Sodiumchloride, NaCl A R.	0.02 g	
Iron (II) sulphate, $FeSO_4$. $7H_2O$ A.R.	0.02 g	
Manganese sulphate MnSO $5H_2O$ A R.	0.02 g	
Agar Oxoid No 1 L11	14 g	
Distilled water (g Millipore or redistilled)	1000 ml	
Solution A	0 1 ml	
Solution B	1 ml	

The dry material under complete medium is weighed out in bulk, water added and the suspension boiled for 2 or 3 minutes with frequent agitation. Solutions—or possibly suspensions—A and B are added just prior to heating in the autocla t 117 C for 8 minutes. The medium is cooled to 55–50° C. 14 cm plates are filled with 55 g melted medium (on balance)

The penicillin in the material nder investigation was inactiv ted by presoaking with the addition of 10 µl penicillinase (Penase LEO 200,000 units/ml) to the test discs

5-FU in serial dilution (15 30 60, 120 and 240 µg ml in serum (not inactivated and inactiv ted for 30 m and 56 C) plasma, urine, bile and saline was used for construction of standard curves (Fig 1 The diameter of the zone of inhibi tion of bacterial growth around a sample was recorded after 20 hours at 37 C (Fig 2) and the concentration of activ drug was calculated by means of standard curves plotted daily

ATCC 8043 and 33 other bacterial strains were tested for sensitivity to 5-FU They consisted of routine stock cultures and freshly-isolated strains from patients. In this screening procedure, only surface seeding on antibiotic sensitivity agar plates and paper discs containing 2 5 mg 5-FU was used. The growth rate of ten of the 5-FU sensitive strains on the uracil-free medium was then tested and compared with the test strain ATCC 8043

RESULTS

Media

Standard bacteriological media could be used for analysis of samples with high con-

tumour response to 5-FU treatment vary very much in different individuals in relation to their liver and kidney function

The difficulties in predicting the effects of a given dose of 5 FU necessitate the development of a method for determining the concentration of active drug in tissue and body fluids. Furthermore, such a method is necessary for evaluation of the rationale of different forms of administration of the drug

A microbiological method for estimation of the concentration of 5 FU was described by *Clarkson et al* (1964) This was claimed to be superior to the spectrophotometric methods available because it determines lower levels of the drug and registers only the biologically active substance. The method is based on the fact that the growth of an enterococcus strain (ATCC 8043–*S faecalis*) is inhibited by 5-FU

The aim of the present work was to report the technical problems associated with microbiological determination of 5 FU in blood urine and bile

MATERIAL AND METHODS

The experiences are based on analysis of 3 764 samples of serum, plasma, urine and bile (1 881 from patients, 1 169 from dogs and 624 from pigs given 5-FU) Commercial 5-FU (fluorouracil, Roche) 50 mg/ml, was used The enterococcus strain ATCC 8043 (*S faecalis*) was used as reference test organism. A total of 34 different bacterial strains were tested

Capps, Hobbs & Fox's Folic Acid Assay Medium was used for determination of 5-FU This was made uracil free and folic acid was added (Table 1) Different types of standard non-selective bacteriological media were also tested. For sensitivity tests a peptonefree agar with 5 per cent horse blood (3) was used Inoculation of the medium with a test strain was performed either by surface seeding or by the pour plate technique. Colonies from an overnight growth were harvested with a 1-microlitre platinum loop suspended in 5 ml nutrient broth and incubated at 37 C for 3 hours. For surface inoculation, 3 ml of this culture in 50 ml sterile distilled water was flooded over the plates and the surplus was drained off carefully The plates were dried at 37 C 90 minutes before and 60 minutes after inoculation. Using the pour-plate technique 30 ml of nutrient broth was inoculated with the bacteria from one colony After incubation for 4 hours at 37 C, the culture was spun down (4 000 rev/min) and the sediment suspended in 10 ml saline. 2 ml of this suspension was mixed thoroughly with 500 ml of medium. Standard plastic Petri dishes (Nunc, Roskilde Denmark) with diameters of 9 and 14 cm, were filled with 25 and 55 g of the medium, respectively on a balance and left to solidify on a carefully levelled table

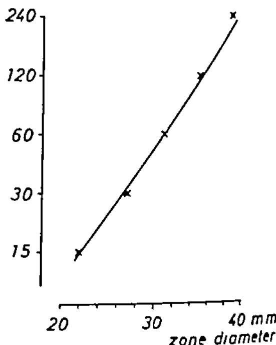


Fig 1 Representative standard curve for 5-FU in human serum.

Three different methods for application of samples were investigated 1) 100 µl of the samples was applied with 50 µl micropipettes into basins (10 mm diameter) cut in the agar plate. The bottom of the basins consisted either of the plastic dish or of agar medium 2) 50 µl of the samples was added to filter paper discs* 10 mm in diameter 3) 10 µl of the samples was added with microcapillar tubes to paper discs 5 mm in diameter* The discs were placed on the agar surface with a needle either directly or after being dried. The plates were either incubated immediately or kept at room temperature for 30 or 120 minutes prior to incubation, to allow evaluation of the influence of prediffusion on the zone of inhibition.

The interaction of some generally used antibiotics and chemotherapeutic drugs on the test system for 5-FU with the enterococcus ATCC 8043 was also investigated, using the agar diffusion paper disc method (AB Biodisk Stockholm) (6)

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TABLE 2. *Inhibition of Different Antibiotics and Chemotherapeutics on the Growth of S. faecalis ATCC 8043 Tested by the Agar Diffusion paper Disc Method (AB Biodisk Stockholm). The Concentrations Tested and those Generally Seen during Routine Therapy and Toxic Concentrations are Given*

Drug	Concentration tested (μg/ml)	Inhibition zone (mm)	Therapeutic conc. (μg/ml) Serum	Urine	Toxic concentration in serum (μg/ml)
Penicillin-G	100	25	10	2000	400
Ampicillin	100	23	10	2000	400
Methicillin	100	<10	10	2000	400
Carbenicillin	1000	21	10	2000	400
Cephalotin	300	<10	15–20	2000	150
Erythromycin	150	29	3	1000	(10–20)
Lincomycin	150	33	5–10	2–200	—
Streptomycin	300	<10	20	1000	20
Kanamycin	300	<10	10–20	200–500	30–40
Gentamycin	300	16	5	100	10
Chloramphenicol	300	38	5	30	30
Tetracycline	300	48	3	300	(20)
Oxytetracycline	300	38	1 2	300	(20)
Doxycycline	300	43	6	30	(20)
Sulfisodomidin	2500	<10	100	1000	150
Nitrofurantoin	300	30	0	100	—
Nalidixin	300	<10	30	250	(50)
Fucidin	300	30	30	0.5	—
Trimethoprim	12	37	0 5–1	200	(1.5)

per cent above the 1000 ng level. The same regression line was obtained irrespective of whether 5-FU was diluted in plasma, urine or saline 1–2 mm smaller and less sharp zones were obtained in inactivated serum, and dilution in bile to 25 ng gave 4–5 mm larger zones. Double inhibition zones were generally seen when testing pig serum (Fig. 3). The outer border was used for registration in these cases. There was no difference between the regression lines obtained by using the serum of the proband or pooled human serum

The medium used was coloured slightly but was transparent. Optimal conditions for the assay were recorded in direct light on the plate with a dark background at about 1 m distance. All the recordings were made at the same angle. When larger zones occurred (>40 mm) dilution of the samples was necessary. 5-FU concentrations were best calculated by using daily plotting of standard curves.

Drug Interaction

The influence of different antibiotics and chemotherapeutics on the growth of *S faecalis* ATCC 8043 can be seen from Table 2. The test strain was not sensitive to sulphonamides, nalidixin, methicillin, streptomycin, cephalotin and kanamycin. The most powerful inhibitors were the tetracyclines, lincomycin and trimethoprim, but penicillins in concentrations of clinical interest also influenced the growth of this strain. By adding 10,000 units penicillinase (LEO) the influence of all types of penicillin and cephalosporins was eliminated. Penicillinase itself had no influence on either the 5-FU the enterococcus strain or the medium.

Inoculation

In order to obtain even growth on the agar surface it was necessary to dry the plates before and after inoculation. To obtain uniform distribution of the bacteria and circu

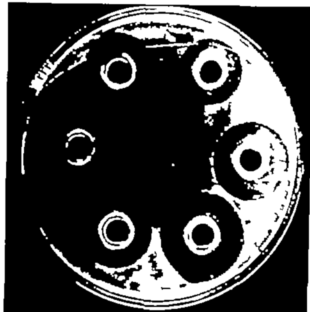


Fig 2 Representative inhibition zones from 5 FU in human serum.

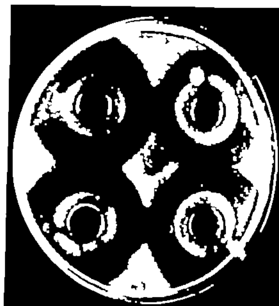


Fig 3 Double inhibition zones from 5-FU in pig serum.

centrations of 5-FU but uracil free medium was necessary to obtain determination of concentrations below 60 ng/ml

Test Strains

The highest sensitivity was shown by *Staphylococcus epidermidis* and *Staphylococcus aureus* and the lowest by *Pseudomonas aeruginosa* and *Salmonella typhimurium*. ATCC 8043 other enterococci pneumococci and haemolytic streptococci had intermediate sensitivity The most distinct inhibition zones caused by 5-FU were obtained in test systems with the enterococci 5 FU was shown to inhibit strongly the *in vitro* growth of bacteria commonly isolated from the respiratory tract The effect on the aerobic normal or faecal flora was less pronounced. The growth rate of ATCC 8043 was intermediate as compared to the ten other 5-FU sensitive strains.

Storage of Samples

No change in activity in human serum containing 5-FU was observed after storage for 24 hours at 20 C (14 samples from 8 patients) In contrast, the 5-FU activity in plasma and serum from dogs diminished by 10 to 15 per cent per 24 hours at +25 C, +4 C, −20 C and +37° C.

Registration

The standard curves were plotted on semi logarithmic paper and linear regression lines were drawn by visual estimation of the range 10–120 ng/ml (Fig 1) Twenty such standard curves were analyzed statistically using Olivetti disc computer P652, and tested against six functions (straight line parabola, cubic exponential power curve and power/exponential) The best fit was found for power curve (r = 0 79) The r value was 0 74 for the exponential function used by us. The methodological error was calculated from 99 duplicate determinations in the present material, using the following formula

$$SE = \sqrt{\frac{\Sigma d_i}{2n}}$$

were d_i is the difference between duplicate determinations (11) The error was then expressed as percentage of the mean of the determinations. Serum from pigs, dogs and humans was included The error was 11.2 per cent within the concentration range 0–120 ng 5-FU/ml serum, 14 4 per cent within the range 120–1000 ng/ml and 15.5

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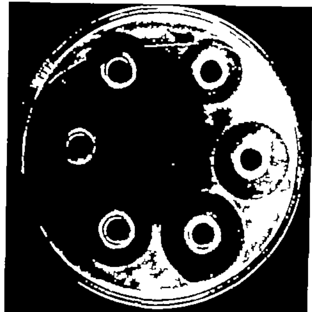


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since it is known that 5-FU concentrations much larger than 250 ng/ml are often encountered during treatment of patients, careful dilution of the sample and continuous use of the basin method seem to be preferable.

Determination of antibiotic activity in human serum is often hampered by an inhibiting property of the serum to the test strain, but this problem was not encountered in the present study with the enterococcus strain against 5-FU.

Dilution of samples could be carried out in serum, plasma, urine or physiological saline. Dilution in bile gave larger inhibition zones, probably because bile served as a detergent and the sample diffused more rapidly in the medium.

Some commonly used antibiotics may interact with the estimation of 5-FU activity. The influence from the penicillin group is easily eliminated by penicillinase. Many other generally used antibiotics have very little influence on the recordings. However, tetracyclines, lincomycin and trimethoprim must be avoided, since it is not possible to eliminate their influence on the test system. As was expected, the enterococcus strain used was not sensitive to sulphonamide and nalidixic acid or to methicillin, streptomycin, cephalotin and kanamycin.

In clinical routine, the practice of storing frozen serum and urine for 24 hours has no disadvantages. However, serum and plasma from dogs are more sensitive to storage and the activity may be reduced by 10–15 per cent during such conditions.

We have used visually estimated regression lines in a semilogarithmic system as standard curves, as has also been done by i.a. *Clarkson et al.* (5) and *Hunt et al.* (8). The statistical analysis shows that this is not quite correct from a mathematical point of view and has thus caused some error in the method. However, the type of reference curve is easy to draw and the methodological error was only 10–15 per cent within the concentration range 10 to 10,000 ng/ml sample. It should be stressed that these results are based also on data collected during the initial phase of our work with the method. We are of the opinion that the precision of the assay can be increased by using the surface-seeding technique for inoculation of ATCC 8043, the basin method for application of fresh samples, and the other optimal conditions mentioned (uracil-free medium, large plates, short storage, etc.)

As has been pointed out previously (8), this microbiological method originally worked out by *Clarkson et al.* (5) is sufficiently sensitive for measuring blood concentrations of antitumour compounds (or cytotoxic equivalents) following 5-FU treatment. The present study draws attention to many factors which influence the recordings. We recommend strongly a laboratory routine which includes, for instance, daily construction of standard curves, using the same batch of inoculated plates and a careful inventory and close registration of drugs which might interfere with the growth of the test strain. According to our experience, the details given in this paper form the basis for successful application of the method for registration of 5-FU activity both in clinical and experimental work.

CONCLUSION

Summarized Instruction for Laboratory Use

Cytostaticum. 5-fluoro-uracil (Roche) 50 mg/ml in 5 ml ampoules. Storage at 25 °C.

Test strain. *Streptococcus faecalis* (ATCC 8043)

Medium. U 348 (Table 1) 55 g medium/plate.

Size of plate. Diameter 14 mm (Nunc, Roskilde, Denmark)

Inoculation. Dry the plates in a dry incubator at 37 °C for about one hour. Take one single colony from an overnight incubated blood-agar plate. Suspend the material in 5 ml of nutrient broth and incubate at 37 °C for 3 hours. Add 3 ml of this culture to 50 ml sterile distilled water. Flood the plates with this suspension. Carefully remove the surplus by suction and dry the

lar zones by the pour plate technique it was necessary to mix thoroughly the bacterial suspension with the melted medium at 48° C. The inhibition zones were smaller with the pour plate technique than with the surface seeding technique

Plate Size

The space for free diffusion of 5-FU (in clinical concentration") was too small in 9 cm plates. Plates with a diameter of 14 cm with only 4–6 samples on each were more suitable. Standardized thickness was achieved by filling 14 cm petri dishes with 55 g of medium on a balance, and leaving them to solidify on a horizontal table.

The plates could be stored for 14 days at +4° C and inoculated plates up to 5 days.

Application of Samples

The inhibition zones were of the same size independent of the two types of basins in the agar Using the two filter paper disc methods, the zone diameters were 10 and 50 per cent smaller respectively If the sample was allowed to dry on the paper disc before being placed on the agar surface the inhibition zones were larger and more distinct

Prediffusion

With prediffusion 1–3 mm larger and sharper inhibition zones were obtained as compared to direct incubation.

DISCUSSION

Sensitivity to 5 FU seems to be a general property of bacteria Certain strains, especially gram negative bacilli such as proteus, pseudomonas, klebsiella *E coli* and salmonella show a rather low sensitivity to 5-FU as compared to enterococci while others like *Staphylococcus epidermidis* are much more sensitive Pneumococci and β haemolytic streptococci have about the same sensitivity to 5-FU as enterococci Judging from the present *in vitro* results, disturbances during treatment with 5 FU may be expected in the normal oral flora but probably not in the aerobic fecal flora.

The enterococcus strain ATCC 8043 (*S faecalis*) is easy to handle and has a suitable growth rate by surface seeding on the uracil-free medium. This method seems superior to the pour plate technique with the medium containing uracil originally used by *Clarkson et al* (5)

Ordinary standard media for bacteriological work are not suitable for registration of the low 5 FU concentrations usually reached continuous infusion of 15 mg 5-FU per kg body weight per 24 hours (1) By making the medium uracil free, it has been possible to obtain a test system which is suitable also for low concentrations of 5-FU It is probable that the repressor system of the bacteria works and the sensitivity becomes higher The bacteria need uracil, and if the medium does not contain this substance they synthesize the enzyme necessary for uracil synthesis. 5 FU seems to inhibit synthesis of both RNA and DNA in bacteria (9) and thus also the synthesis of enzymes. This is in contrast to the conditions in mammalian cells, where mainly DNA-synthesis is involved.

The present investigation has shown that the medium and the surface-seeding method with ATCC 8043 used are easy to handle. The method is more sensitive and results in only minor methodological errors as compared to the pour plate method.

We have found that agar plates of at least 14 cm in diameter must be used to permit free diffusion and to eliminate the risk of an influence between materials from the sources of diffusion. Furthermore the plates must have a constant agar thickness and this can be accomplished by weighing the plates during pouring We have also found cylindrical holes cut in the agar to be suitable for routine use. They permit exact application also of voluminous samples, which increases the sensitivity of the assay The rather large agar plate permits direct recording of 4-6 samples containing up to 250 ng 5-FU/ml (Fig 1) It is true that the filter paper disc method gives smaller inhibition zones, but

Acta path. microbiol. scand. Sect. B 85: 235–237, 1977

BRIEF REPORTS

T-TYPING OF GROUP A STREPTOCOCCI FROM CLINICAL SPECIMENS RESTRICTION OF THE NUMBER OF IMPLIED M TYPES IN EACH T PATTERN BY TESTS FOR OPACITY FACTOR AND NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE

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Larsson, R. & Christensen, P. T-typing of group A streptococci from clinical specimens: restriction of the number of implied M types in each T-pattern by tests for opacity factor and nicotinamide adenine dinucleotide glycohydrolase. Acta path. microbiol. scand. Sect. B, 85: 235–237, 1977.

117 group A streptococci, consecutively isolated from routine throat swab cultures, were T-typed and further characterized according to their enzyme reactions. The results of typing with two different commercial kits of anti-T sera showed good agreement. 89 per cent of the strains were typable by either of these kits. 68 per cent of the strains were OF-positive and 95 per cent NADase-positive. With T-typing alone, 22 per cent of the strains showed agglutination patterns implying only one M type each. When considered together, the T-typing and enzyme reactions made it possible to implicate one M type only for each of 45 per cent of the strains. In further 34 per cent of the strains, the possible M type of each strain was restricted to four types. However, these findings were hampered by the fact that the T patterns of a number of more seldom met group A streptococci has not been investigated adequately.

Key words: Group A streptococci; T-typing; M types.

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Received 28.ii.77   Accepted 28.ii.77

The clinical and epidemiological significance of typing group A streptococci is well established. Typing is especially useful for charting epidemics of acute post-streptococcal nephritis, since only a few types have been connected with this disease. As far as concerns the throat strains, type M 12 (but also M types 4, 6 and 25) is potentially nephritogenic (8).

There are several reasons why many clinical laboratories prefer T typing to M typing, although the final type is determined by the more specific M antigens (5). Above all, up to 90 per cent of a clinical unselected collection of strains can be T typed, whereas no more than 50 per cent can be M typed (5). Furthermore, there are commercial anti-T sera available, but no anti-M sera. The observation that the opacity factor (OF) and nicotinamide adenine dinucleotide glycohydrolase (NADase) is restricted to certain M types made it possible to divide T patterns further into subgroups (see Ofek et al. (7)).

The present work concerns the application of T typing and OF and NADase production on 117 group A streptococcal strains, isolated in our laboratory from throat specimens.

Materials and Methods

Strains. Group A streptococci, 117 strains consecutively isolated from throat swab specimens sent to our laboratory between the 1st and 15th November 1976, were investigated.

Serological Grouping. Group A streptococci were

plates again in the dry 37° C incubator for 90 minutes

Application of the sample Cut holes in the agar with a sharp-edged metal tube (diameter 10 mm) not more than 4 basins per plate Remove the agar in the hole with a cannula. Apply 0 1 ml of a sample with a micropipette into two opposite basins. Do not use any prediffusion time. Incubate at 37° C for about 20 hours. Record the outer most inhibition zone

Serial dilution of 5 FU Use distilled water for the dilution of 5-FU to the concentration levels of the regression line (<500 ng/ml) Use non-inactivated pooled human serum for 500 250 125 60 30 15 and 7.5 ng

Media. The medium used is extremely sensitive to high temperature. Good plates have a light yellow colour Ready plates can be stored at 4° C for 14 days and inoculated plates for 5 days.

This work was supported by the *Swedish Cancer Society* (Project No 512 B71-OIX)

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TABLE 3 *T Types and Enzyme Reactions of 117 Group A Streptococcal Strains Isolated from Throat Cultures*

T pattern	N of strains	M types implicated*	SOR	NADase	No. of strains	M types implicated*
1	6	M1	—	—	6	M1
2	16	M2	+	+	14	M2
			—	+	2	
3/13/B_{3264}	10	M3 13 33 39 41 43 52 53 56				
			+	+	6	M13
			—	+	4	M3
4/28	52	M4 24 26 28 29 46 48 60				
			+	+	40	M4 28, 48, 6..
			—	+	12	?
6	4	M6	—	+	4	M6
12	16	M12, 22	+	+	10	M22
			—	+	6	M12
Not typable	13	–	+	+	7	–
			—	+	6	

The T antigens of M types 32, 34 36 37 38, 40, 42 50 62, and 63 have not been adequately investigated.

emerging from the present work was that it was possible to relate the group A streptococci to a restricted number of M types, by T typing and enzyme reactions In 45 per cent of the strains, one M type was implicated for each strain by this method and in a further 34 per cent, four different M types for each strain. There remains to be described, however a simple method by which the M types can be definitively defined in strains from clinical specimens It is possible that the type-specific SOR-inhibiting sera described by *Maxted et al* (6) could provide such a tool in clinical routine

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TABLE 1 *Streptococcal Anti-T Sera (Chemapol) Used in the Investigation*

Polyvalent antiserum pools	Constituting monovalent sera
T	1 3 13 B_{3264}
U	2 4 6 28
W	5 11 12 27 44
X	8, 14 25 Imp 19
Y	9 18 22 23

initially recognized by bacitracin sensitivity (4) and increased hemolysis in the presence of nucleate (2) as described by *Wallerström* (10) Definitive serological grouping was performed as described previously (1)

T typing Two sets of commercial antisera were used one set, purchased from Chemapol Prague contained the antisera indicated in Table 1 The other set was manufactured by Toshiba Kagaku Kogyo Co., Ltd., Tokyo and was very kindly donated by Dr *Y Miyamoto* Kanagawa Prefectural Public Health Laboratory Japan. The sera were named as listed in Table 1 with the following exceptions the monovalent sera 5 27 and 44 were supplied as a single serum, 5/27/44 and anti T 14 was named T14/49

Tests for OF and NADse OF was demonstrated as described by *Maxted et al.* (6) (the "plate method") The strains were investigated for NADse production as described by *Lütticken et al* (3)

Results and Discussion

Among the 117 strains of group A streptococci investigated 102 (89 per cent) were T typable with the T typing set from Chemapol (Table 2) T type 4 dominated, constituting one third of the strains, followed by T2 T12 and T28 14 14 and 11 per cent, respectively There was good agreement between the results obtained with the two sets of commercial antisera the same types were obtained, with the exception that one strain, non-typable with the Chemapol sera was T13 with the other set, while a T4 typed by the Chemapol sera was non-typable with the Japanese sera. This correlation was somewhat surprising, considering the results reported of a comparison of strains between different laboratories (9)

TABLE 2 *T Patterns of 117 Group A Isolated from Throat Cultures*

T pattern	Number of strains
1	6
2	16
3/13/B 3264	6
4	39
6	4
12	16
13	4
28	13
Not typable	13

It is not possible to "translate" T types to M types direct partly because a number of M types have not been adequately investigated with respect to T antigens (5) (see Table 3) There are two main possible reasons for this lack of information (I) many of these strains are very seldom met with in clinical specimens, and (II) recently described, "new" M types (for example types M 62 and M63)) mainly impetigo strains, have not been sufficiently characterised

The type T1 T2 and T6 strains isolated constituted 2? per cent of the group A streptococci investigated the M types implicated in these T patterns are types M1 M2 and M6 respectively (5) with the limitations described above it was thus possible to connect one-fifth of the strains with one probable M type on the basis of T typing (Table 3)

68 per cent of the strains were OF positive while 95 per cent were NADse-positive the NADse-negative strains were all T1 Thus, under the epidemiological circumstances during which the strains were collected, OF-reaction was more useful than NADse-production for the distinguishing of different T patterns.

Based on a background knowledge of the distribution of OF (see *Maxted et al.* (5) and NADse production (*Ofek et al.* (7) with the corrections of *Lutticken et al* (3) among the various M types, it was possible to reduce still further the number of M types implicated in each T pattern found (Table 3) In 44 per cent of the strains the T pattern and enzyme reactions found attributed the strain to a single M type. For example the enzyme reactions divided the T12 pattern into two groups (I) 10 strains probably related to M22 but with certainty not to M12 and (II) 6 other strains, the T pattern and enzyme reactions of which implicated M12

The T4/28 complex represented 52 strains, among which the enzyme reactions of 12 could not attribute the strains to the reactivity of any known M type

To our knowledge no detailed study has yet been made of the genetics of OF and NADse characteristics. However *Maxted et al.* (6) stated that the OF is a stable property of group A streptococci and the investigations of *Ofek et al.* (7) and others (see *Lutticken et al.* 3) indicated the same as regards NADse production The main point

TABLE 1 *Statistical Comparison by Use of 2 × 2 Contingency Tables, of Ochratoxin Contamination of Cereals from an Endemic Area (EN) and Non-endemic Area (Non-EN) Yates Correction for Continuity is Used*

	Non-EN	EN	Total
Maize			
No. of samples contaminated	1	2	3
No. of samples not contaminated	37	8	45
Total	38	10	48
Per cent contaminated	2.6	20.0	
Wheat			
No. of samples contaminated	0	3	3
No. of samples not contaminated	12	25	37
Total	12	28	40
Per cent contaminated	0	10.7	
Barley			
No. of samples contaminated	0	1	1
No. of samples not contaminated	14	8	22
Total	14	9	23
Per cent contaminated	0	11.1	
All cereals			
No. of samples contaminated	1	6	7
No. of samples not contaminated	63	41	104
Total	64	47	111
Per cent contaminated	1.6	12.8	
$\chi^2 = 4.02 - P < 0.05$			

TABLE 2. *Concentration of Ochratoxin A Foodstuff*

	μg ochratoxin A/kg
Endemic area	
Maize	18, 90
Wheat	12, 36, 55
Barley	5
Pork	5
Non-endemic area	
Maize	14

One sample out of 12 pork samples was contaminated with ochratoxin A. No pork samples from the non-endemic were collected.

Materials and Method

Sampling of foodstuff was conducted in two areas near Slavonski Brod, Yugoslavia, in the period 1971 1975. One endemic area, Kanda, and one non-endemic area (control area) Klakar Pol. Sewete. The prevalence of EN in the endemic area (Kanda) is well documented 7.3 per cent of the population was affected by EN in 1974 and 4.3 per cent was found suspect of EN (2). In Klakar Pol. Sewete no cases of EN have been observed. Samples (500 g) of homeproduced foodstuffs (grains, pork) were drawn randomly from household stocks. Analytical samples (50 g) were subjected to thin-layer chromatographic analysis for ochratoxin (6). Positive TLC results were confirmed by derivative formation. The frequencies of ochratoxin contamination in food stuffs from endemic and non-endemic areas were compared by use of 2 × 2 contingency tables (10).

Results and Discussion

Ochratoxin A was found in foodstuffs from both endemic and non-endemic areas. However the frequency of ochratoxin contamination in cereals was significantly higher in the endemic area compared to the non-endemic area (Table 1).

Preliminary results of on-going investigations (7) involving huge numbers of foodstuffs, confirm the high contamination frequency observed in the endemic area (Kanda). The comparatively low frequencies of ochratoxin A contamination in foodstuffs from the non-endemic area are similar to the range found in countries where EN has not

Acta path. microbiol. scand. Sect. B 85 238–240 1977

BALKAN (ENDEMIC) NEPHROPATHY AND FOODBORN OCHRATOXIN A PRELIMINARY RESULTS OF A SURVEY OF FOODSTUFFS

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Krogh, P. Hald, B. Pleština, R. & Ceović, S. Balkan (endemic) nephropathy and foodborn ochratoxin A. Preliminary results of a survey of foodstuffs. Acta path. microbiol. scand. Sect. B 85 238–240 1977

Ochratoxin A is a nephrotoxic fungal metabolite (mycotoxin) occurring in foodstuffs. The compound is causally associated with mycotoxic porcine nephropathy, a disease comparable with a human kidney disease, Balkan endemic nephropathy. A preliminary survey of home-produced foodstuffs in areas of Yugoslavia revealed that contamination with ochratoxin A is more frequent in an area where Balkan endemic nephropathy is prevalent (endemic area) than in an area where this disease is absent. This indicates higher exposure to foodborn ochratoxin A in the endemic area. Thus further evidence is provided supporting the hypothesis that ochratoxin A is a disease determinant of Balkan endemic nephropathy.

Key words: Ochratoxin A; mycotoxin; Balkan endemic nephropathy.

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Received 3.v.77   Accepted 3.v.77

Balkan (Endemic) Nephropathy (EN) is a fatal chronic kidney disease affecting rural populations in areas of Bulgaria, Romania, and Yugoslavia. The etiology of the disease is still unknown, after more than 20 years of etiological investigations which have included the search for heavy metals, trace elements, infectious agents (bacteria, virus) and genetic factors (8).

Mycotoxic nephropathy is a naturally occurring disease so far observed in pigs and poultry. Ochratoxin A, a dihydro-isocoumarin derivative linked through its 7-carboxy group to L-β-phenylalanine, is a nephrotoxic secondary metabolite (mycotoxin) of several species belonging to the fungal genera *Aspergillus* and *Penicillium* (4). This compound, occurring in feeds and foods, has been observed as a major disease determinant of porcine and possible also avian nephropathy (4). *Krogh* (3) called the attention to the striking similarities in the changes of renal structure and function between EN and ochratoxin A-induced porcine nephropathy suggesting common causal relationships. In addition several epidemiological similarities between the human and porcine nephropathy have been observed including the endemic occurrence (5). In order to elucidate the possible involvement of ochratoxin A as a disease determinant of EN, investigations were initiated aiming at the determination of ochratoxin exposure through foodstuff in endemic and non-endemic areas.

TABLE 1 *Statistical Comparison by Use of 2 × 2 Contingency Tables of O hratoxin Contamination of Cereals from an Endemic Area (EN) and Non-endemic Area (Non-EN) Yates Correction for Continuity is Used*

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No of samples not contaminated	14	8	22
Total	14	9	23
Per cent contaminated	0	11 1	
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No. of samples contaminated	1	6	7
No of samples not contaminated	63	41	104
Total	64	47	111
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Pork	5
Non-endemic area	
Maize	14

One sample out of 12 pork samples was contaminated ith ochratoxin A No pork samples from the non-endemic were collected

Materials and Methods

Sampling of foodstuffs was conducted in two areas near Slavonski Brod Yugosla ia, in the period 1971 1973 One endemic area, Kamla, and one non-endemic area (control area) Klakar Pol. Servete The prevalence of EN in the endemic are (Kamla) is well documented 7 3 per cent of the population was affected by EN in 1974 and 4 3 per cent was found suspect of EN (2) I Klakar Pol Servete no cases of EN hav been observed. Samples (500 g) of homeproduced foodstuffs (grains, pork) were drawn randomly from household stocks. Analytical samples (50 g) were subjected to thin-layer chromatographic analysis for ochratoxin (6) Positiv TLC results were confirmed by deri tive formation. The frequencies of ochratoxin contamination in foodstuffs from endemic and non-endemic areas were compared by use of 2 × 2 contingency tables (10)

Result and Discussion

Ochratoxin A was found in foodstuff from both endemic and non-endemic areas. However the frequency of ochratoxin contamination in cereals was significantly higher in the endemic area compared to the non-endemic area (Table 1)

Preliminary results of on-going investigations (7) involving huge numbers of foodstuffs, confirm the high contamination frequency observed in the endemic area (Kamlfa) The comparativ ly low frequencies of ochratoxin A contamination in foodstuff from the non-endemic area re simila to the range found in countries where EN ha not

been reported. Thus 2.6 per cent of maize in France was found contaminated in 1974 (1) and 0–2.8 per cent of wheat in USA contained ochratoxin A in the period 1970–1973 (9)

The concentration of ochratoxin A in grains ranges from 5 to 90 μg/kg (Table 2) In addition *5 μg/kg of ochratoxin A was found in one* out of twelve pork samples collected in the endemic area. This occurrence is probably the result of the use of ochratoxin contaminated animal feed, pointing to residues of ochratoxin A in tissues of slaughter animals (pig poultry) as an important exposure channel (4)

The higher frequency of ochratoxin A contamination of foodstuffs indicates higher ochratoxin A exposure to humans in the endemic area. Thus further evidence is provided supporting the hypothesis that ochratoxin A is a disease determinant of Balkan endemic nephropathy

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This investigation received financial support from the *World Health Organization* the *Danish International Development Agency* and the *Research Council of SR Croatia* The authors are indepted to professor *M Šarić* Institute for Medical Research and Occupational Health, Zagreb, and to director *I Grgić* Medical Centre Slavonski Brod, for valuable assistance

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Acta path. microbiol. scand. Sect. B, 85: 241–248, 1977

CYTOCHEMICAL ELECTRON MICROSCOPY ON POLYSACCHARIDE GRANULES IN THE ENDOGENOUS FORMS OF *EIMERIA BRUNETTI*

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Ferguson, D. J. P., Birch-Andersen, A., Hutchison, W. M. & Siim, J. Chr. Cytochemical electron microscopy on polysaccharide granules in the endogenous forms of *Eimeria brunetti*. Acta path. microbiol. scand. Sect. B, 85: 241–248, 1977.

The endogenous stages in the life cycle of *Eimeria brunetti* were tested for polysaccharide material by electron microscopical cytochemistry using the periodic acid-thiocarbohydrazide-osmium tetroxide (PATO)-method. The parasites were observed within the epithelial cells of the small intestine of infected domestic fowls. Two types of granules reacted positively for polysaccharide. The first was large, approximately 500 nm by 250 nm. These granules had an appearance similar to the polysaccharide granules reported for other coccidian parasites. They were observed in mature merozoites, macrogamonts, and developing oocysts. The second type of granule was smaller (15–30 nm in diameter) and was only observed at the periphery of the residual cytoplasmic mass of mature microgamonts. It was more similar in appearance to metazoan glycogen than the former. However, since the PATO-method does not differentiate between different polysaccharides it can not be proven if these granules in fact contain glycogen. The WFB I of the macrogamete and the outer layer of the oocyst wall gav[e a] slight positive reaction but after examination of the controls it appeared that this was not a specific reaction for polysaccharides.

Key words: *Eimeria brunetti*; cytochemistry; electron microscopy; polysaccharide granules; chickens.

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Received 9.ii.77. Accepted 9.ii.77.

Work initiated while a Wellcome Trust Travelling Research Fellow and completed as a Danish Medical Research Council Fellow.

Polysaccharide granules have been found in many stages of the life cycle of parasites belonging to the coccidia. The polysaccharide was originally thought to be glycogen but

electron microscopy showed that the ultrastructure of the granules was different from that of metazoan glycogen particles. In consequence of this, *Scholtyseck* (11) proposed the term "Coccidienglykogen" for the polysaccharide present in the granules of the coccidians. In biochemical studies the polysaccharide of the coccidia was isolated and found to be amylopectin (10). *Ryley* (9) has reviewed the cytochemical as well as the biochemical aspects of the polysaccharides present in coccidians.

In previous studies (3, 4, 5 & 6) on the ultrastructure of the endogenous development of *Eimeria brunetti* (a pathogenic coccidian of domestic fowls) we have referred to the presence or absence of polysaccharide granules. The identifications were, however, based solely on the morphological features of the granules as described for other coccidian parasites (12). Recently we have used the periodic acid-thiocarbohydrazide-osmium method (7 & 14) for the demonstration of polysaccharides in thin sections of the endogenous forms of *E. brunetti*, thus attempting a histochemical corroboration of our morphological evidence. Our results are presented in this report.

MATERIALS AND METHODS

Tissue Preparation

The techniques used to prepare the endogenous forms for ultrastructural examination have been previously described (3), but in brief pieces of the small intestine of infected chickens were fixed in glutaraldehyde and osmium tetroxide and embedded in Vestopal W.

Histochemical Technique

Crystalline periodic acid (analytical reagent) and thiocarbohydrazide for electron microscopy were obtained from E. Merck, Darmstadt, West Germany. Osmium tetroxide was supplied by W. C. Heraeus, Hanau, West Germany.

The test for polysaccharides was carried out using a variation of the periodic acid-thiocarbo-

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Figures 1–12 are micrographs obtained from sections of epithelial cells of the small intestine of chickens infected with *Eimeria brunetti*. All sections used for illustrations have been post-stained with magnesium uranyl acetate and lead citrate.

The following abbreviations are used throughout: C – Canaliculi; ER – Rough Endoplasmic Reticulum; FL – Flagellum; I – Inner Layer of the Oocyst Wall; LM – Limiting Membrane of the Cytoplasmic Mass; M – Microneme; MG – microgamete; MI – Mitochondrion; N – Nucleus; O – Outer Layer of the Oocyst Wall; PG – Polysaccharide Granule; R – Rhoptry; RM – Residual Cytoplasmic Mass; RN – Residual Nucleus; V – Outer Veil of Oocyst Wall; WFB I – Wall-Forming Bodies of Type I; WFB II – Wall Forming Bodies of Type II.

Double or single bars on the micrographs represent 1 µm or 100 nm, respectively.

Fig. 1. A section through a merozoite which has been treated by the PATO-method showing the positive polysaccharide granules. The nucleus, rhoptries, micronemes, and a mitochondrion are also visible. × 30 000

Fig. 2. A control section showing part of a macrogamete which has not been reacted with any of the chemicals of the PATO-method. The nucleus, polysaccharide granules, mitochondria, WFB I and WFB II can be seen. × 15,000

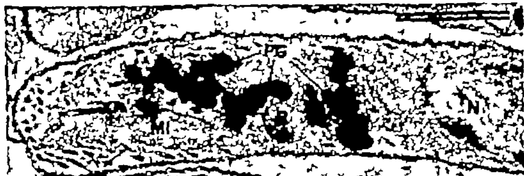
Fig. 3. Part of a macrogamete which has been treated by the PATO-method showing reaction products on the polysaccharide granules. Note that the WFB I appears to have reacted slightly (as evidenced by the increased density to electrons), while the WFB II shows no reaction. × 15 000

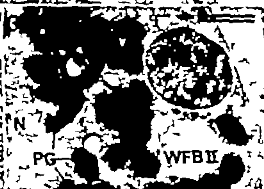
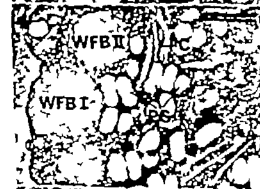
Fig. 4. A control section showing part of a macrogamete which has only been treated with the osmium tetroxide step of the PATO-method. Note that no reaction is shown by the polysaccharide granules, the WFB I or the WFB II. × 15,000.

Fig. 5. An unoxidized control section through part of a macrogamete which has been treated with the thiocarbohydrazide and osmium tetroxide steps of the PATO-method. Note that the polysaccharide granules show no reaction, but that the increase in density of the WFB I in comparison to the WFB II is retained. × 15 000

Fig. 6. Part of an early macrogametocyte which has been treated by the PATO-method. The small, newly formed polysaccharide granules show a positive reaction. The nucleus, rough endoplasmic reticulum, WFB I and WFB II of the organism are also shown. × 30 000

Fig. 7. Part of a section through a fully formed oocyst which has been treated by the PATO-method. Note the positive reaction of the polysaccharide granules and the seemingly slightly positive reaction of the outer layer of the oocyst wall. × 45,000


1
PG
N
MI

2
WFB II
PG
N
MI
WFB I

3
N
PG
WFB II

4
WFB II
C
WFB I
PG

5
N
WFB II
C

6
N
WFB II
WFB I
PG
ER

7
O
PG

electron microscopy showed that the ultra structure of the granules was different from that of metazoan glycogen particles. In consequence of this, *Scholtyseck* (11) proposed the term "Coccidienglykogen" for the polysaccharide present in the granules of the coccidians. In biochemical studies the polysaccharide of the coccidia was isolated and found to be amylopectin (10). *Ryley* (9) has reviewed the cytochemical as well as the biochemical aspects of the polysaccharides present in coccidians.

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Fig. 2. A control section showing part of a macrogamete which has not been reacted with any of the chemicals of the PATO-method. The nucleus, polysaccharide granules, mitochondria, WFB I and WFB II can be seen. × 15 000.

Fig. 3. Part of a macrogamete which has been treated by the PATO-method showing reaction products on the polysaccharide granules. Note that the WFB I appears to have reacted slightly (as evidenced by the increased density to electrons), while the WFB II shows no reaction. × 15 000.

Fig. 4. A control section showing part of a macrogamete which has only been treated with the osmium tetroxide step of the PATO-method. Note that no reaction is shown by the polysaccharide granules, the WFB I or the WFB II. × 15,000.

Fig. 5. An unoxidized control section through part of a macrogamete which has been treated with the thiocarbohydrazide and osmium tetroxide steps of the PATO-method. Note that the polysaccharide granules show no reaction, but that the increase in density of the WFB I in comparison to the WFB II is retained. × 15 000.

Fig. 6. Part of an early macrogametocyte which has been treated by the PATO-method. The small, newly formed polysaccharide granules show a positive reaction. The nucleus, rough endoplasmic reticulum, WFB I and WFB II of the organism are also shown. × 30 000.

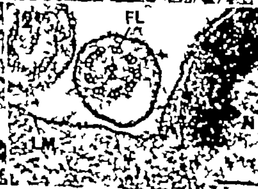
Fig. 7. Part of a section through a fully formed oocyst which has been treated by the PATO-method. Note the positive reaction of the polysaccharide granules and the seemingly slightly positive reaction of the outer layer of the oocyst wall. × 45,000.

8
FL
RN
RM
MG
PG
MG









hydrazide-osmium tetroxide (PATO) method introduced by *Hanker et al* (7) and *Seligman et al.* (14) *Thiéry* (15) evaluated this method and compared the osmium black deposits obtained with the deposits developed with a silver proteinate reagent instead of osmium treatment. Dr *J Theilade** has modified Thiéry's schedule for the PATO-method and has obtained reproducible results for the demonstration of polysaccharides in ultrathin sections of human dental plaques. He was most generous in placing his protocol at our disposal and the procedure we have used is almost identical to the one developed in his laboratory The details are as follows

Sections are placed on formwar coated carbon reinforced 200 mesh gold grids. Through all reagent baths and washing procedures the grids are carried in BEEM capsules (Better Equipment for Electron Microscopy Inc., Bronx, N Y USA) from which the tips have been cut off to leave a hole for draining The BEEM capsules hang by their open lids on the edges of plastic tubes (Lusteroid tubes, Spinco Division, Beckman Instruments Inc. Belmont California) which have been cut down to a volume of about 2–2½ cc. Prior to use capsules and tubes are treated with 0 1 M HCl in 70 per cent alcohol and thoroughly rinsed in 100 per cent alcohol. The following reagents are prepared 1 per cent (W/V) periodic acid in distilled water prepared approximately half an hour before use 1 per cent (W/V) thiocarbohydrazide in 5 per cent acetic acid prepared at 60 C with stirring for 15 min (magnetic stirrer) and stored overnight at 60 C before use. 2 per cent (W/V) osmium tetroxide in distilled water prepared 1–2 days before use. Grids are treated at room temperature (20–22 C) with all reagents, except the thiocarbohydrazide according to the following schedule

1) 1 per cent periodic acid for 45 min in a dark room.
2) The capsules with grids are washed in a jet of distilled water using a volume of approximately 10–15 cc and then allowed to soak in water for 5 min. This procedure is repeated and followed by a final wash with 25 cc of water
3) The capsules are drained with filterpaper and transferred to the thiocarbohydrazide solution which is millipore-filtered (0 45 μ pore size) immediately before use treatment for 2 hours at 60 C.
4) The previously mentioned washing procedure repeated
5) The osmium tetroxide treatment is carried out for 2 hours in a fume hood
6) Finally the grids, still in the BEEM capsules, are washed as previously described

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Six grids with sections were used for each experiment in order to include the necessary controls, whereby treatment with one of the reagents was omitted to detect any false positive reactions. Each series was carried out in duplicate and one grid was examined directly while the other was post-stained with magnesium uranyl acetate and lead citrate Electron microscopy was performed as previously described (3) but to allow easier identification of organelle structure only micrographs obtained from post-stained grids were used for the illustrations of this report.

RESULTS

When thin sections of the endogenous forms of *E brunetti* were cytochemically tested for polysaccharides, two types of granules showed a positive reaction

The first type of granule was a large structure which was observed in the mature me-

Fig 8 A section through a late microgamont which was treated by the PATO-method The small granules with reaction products in the peripheral cytoplasm of the residual cytoplasmic masses are evident. Some developing microgametes can also be seen. × 15 000

Fig 9 An enlargement of part of a small region of a microgamont in Fig 8 in which part of a microgamete is shown. Note that the polysaccharide granules with reaction products are present only below the limiting membrane of the residual cytoplasmic mass. × 90 000

Fig 10 An untreated control section through part of a late microgamont, and a developing microgamete. Note the small glycogen-like polysaccharide granules distinguishable adjacent to the limiting membrane of the residual cytoplasmic mass. × 90 000

Fig 11 An unoxidized control section showing part of a late microgamont which has been treated with the thiocarbohydrazide and the osmium tetroxide steps of the PATO-method. Part of a microgamete is also seen Note particularly the ghost images of the polysaccharide granules present adjacent to the limiting membrane of the residual cytoplasmic mass of the microgamont. × 90 000

Fig 1 A section showing part of an early microgamont which has been treated with the PATO-method. A nucleus can be seen protruding from the surface of the microgamont. Note the lack of any reactive polysaccharide granules at this stage of development. × 90 000

of *E. brunetti*. To the best of our knowledge, though, it has not been reported for other members of the coccidia. However unless specific polysaccharide staining methods are used, the granules could easily have been overlooked in other species.

These small granules in the late microgamont could be related to the energy requirements of the microgamete. The microgametes must have a high energy requirement to power their two large flagella and thus chemical energy will have to be present in the microgamete prior to its release from the microgamont. The small glycogen-like polysaccharide granules may represent a surplus of an intermediate metabolic product which is participating in the provision of the necessary energy for the mature microgamete.

The WFB I, called plastic granules by some authors, and the oocyst wall of *E. brunetti* have been reported as being positive for both polysaccharide and protein in a cytochemical light microscope study (8). In addition, in a cytochemical electron microscope study of *E. tenella* the WFB I and the outer layer of the oocyst wall were reported to give a positive reaction for polysaccharides (2). In our experiments, these bodies and the outer layer of the oocyst wall also appeared slightly positive after the complete PATO-treatment, but a similar increase in density to electrons was seen in controls which had not been oxidized with periodic acid. This indicates that constituents in the WFB I and outer layer of the oocyst of *E. brunetti* other than periodic acid reactive polysaccharides (e.g. proteins) must be responsible for the kind of reaction we observed. This result is fully in line with the results of other studies from which it was concluded that unoxidized controls are necessary for a valid cytochemical interpretation of the demonstration of polysaccharides at the ultrastructural level (1).

We are indebted to the *Central Veterinary Laboratory*, Ministry of Agriculture, Fisheries and Food, New Haw, Weybridge, Surrey, England, for supplying pure sample of oocysts of *E. brunetti*, and to *K. L. Fennestad* V.M.D., Statens Seruminstitut, for provision and maintenance of the chickens. We gratefully acknowledge Mrs. *H. Rasn* and Mrs. *J. Berg* for technical assistance, and Miss *A. Overgaard* and Mr. *F. Larsen* for photographic assistance.

The work was supported by grants from the *World Health Organisation*, Geneva, *The Wellcome Trust*, the *British Council*, the *Danish Medical Research Council* and the *W. H. Ross Foundation*.

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rozoite where it appeared spherical and was approximately 250 nm in diameter (Fig 1) It was also present in the macrogamete and in developing oocysts where it appeared ovoid and measured approximately 500 nm by 250 nm (Fig 3) This type of granule appeared to develop from structures approximately 130 nm by 40 nm observed between strands of the rough endoplasmic reticulum (Fig 6) in the early macrogamont, in the late schizont and in the forming merozoite Only a few of the large polysaccharide granules were present in all the generations of the merozoites but large numbers were observed in the macrogametes and the developing oocysts. In the microgamont, however none of these large granules were ever observed (Figs. 8–12)

The second type of polysaccharide granule was observed only in late microgamonts and measured approximately 15–30 nm in diameter (Figs. 8 & 9) It is thus considerably smaller in size than the polysaccharide granules previously mentioned Large numbers of this smaller granule were distributed at the periphery of the residual cytoplasmic mass of the late microgamont (Fig 8) The cytoplasm of organisms at earlier developmental stages in microgametogenesis was negative for these granules (Fig 12) The small granules can also be observed in sections of late microgamonts which were treated according to normal sectioning routine including post staining (Fig 10) but prior to our experiment with the PATO method their polysaccharide content was unknown and unsuspected In controls in which only the periodic acid treatment of the PATO-method had been omitted it was possible to identify negative images of these small polysaccharide granules (Fig 11)

The wall forming bodies of Type I (WFB I) in the macrogamete and the developing oocyst appeared to show a faint reaction for polysaccharide when grids which had received the complete treatment were compared to untreated controls (cf Figs. 2 & 3) However in unoxidized control sections which were treated with thiocarbohydrazide and osmium tetroxide this faint reaction persisted (Fig 5) but in unoxidized control sections treated only with osmium tetroxide no reactivity was observed (Fig 4) A previous study (6) has shown that the WFB I give rise to the outer layer of the oocyst wall and this layer also shows the same faint reactivity as the WFB I (Fig 8) We conclude that the WFB I and the outer layer of the oocyst wall are showing a thiocarbohydrazide/osmium reactivity rather than a polysaccharide reactivity

DISCUSSION

The polysaccharide material present in the oocysts of *E. brunetti* has been isolated and characterised as amylopectin (10) The large polysaccharide granules observed, in this study in developing oocysts, macrogamonts, and merozoites would thus appear to be amylopectin The ultrastructure of these granules is similar to that observed for the polysaccharide granules in other members of the coccidia (12) for which it has been proposed that amylopectin is the form of polysaccharide stored by these organisms (9) Similar suggestions have also been advanced for the gregarines which are a related group of organisms (13)

It would appear that the ultrastructure of the polysaccharide granules is related to their chemical composition i.e. the polysaccharide granules of members of the coccidia were reported as being different from metazoan glycogen (Coccidienglykogen) by *Scholtyseck* (11) prior to the granules being characterized as amylopectin (10) This being the case, it would seem that the small polysaccharide granule which we have demonstrated below the limiting membrane of the residual cytoplasmic mass of the microgamont resembles metazoan glycogen rather than amylopectin. The size and shape of the osmium black deposits on these granules at least correspond reasonably well with β-glycogen rosettes demonstrated for example in hepatocytes (15) but it should be emphasized that the PATO-method does not differentiate between any of the periodic acid reactive polysaccharides. It is unknown if this small type of polysaccharide granule is limited to the microgamonts

Acta path. microbiol. scand. Sect. B, 85: 249–254, 1977

DIFFERENTIAL STAINING OF BACTERIA IN CLINICAL SPECIMENS USING ACRIDINE ORANGE BUFFERED AT LOW pH

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Kronvall, G. & Myhre, E. Differential staining of bacteria in clinical specimens using acridine orange buffered at low pH. Acta path. microbiol. scand. Sect. B, 85: 249–254, 1977.

Optimal conditions for acridine orange staining of air dried and methanol fixed bacteria on glass slides were studied. The pH of the staining buffer did not influence the fluorescence of an *S. aureus* and an *E. coli* strain at dye concentrations of 25–50 mg per litre. 81 bacterial strains representing 15 different species were stained with acridine orange under standard conditions, all strains showing orange fluorescence. The pH of the buffer influenced markedly the staining patterns of human cells and tissue materials, as represented by smears of peripheral blood, buccal scrapings, urethral secretions and tracheal exudates. The fluorescence obtained ranged from low intensity green at low pH values to bright orange at neutral and alkaline pH. This variability indicated a possibility of designing conditions for a differential staining method for the detection of bacteria in clinical specimens. The differential staining effect with a low pH in the buffer was confirmed on smears of buccal scrapings, cerebrospinal fluid samples and urethral secretions, showing orange fluorescence of the bacteria present and green-to-yellow fluorescence of background material, cells and tissue debris.

Key words: Differential staining; bacteria; acridine orange; low pH.

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Received 4.i.77. Accepted 4.ii.77

The Gram stain was originally developed as a differential staining method for the detection of pneumococci in clinical specimens, this giving a dark bluish coloration of the bacteria in good contrast with the tissue material. However, this differential staining effect is obviously limited to Gram positive microorganisms. No staining method is yet available which will give one colour for bacteria and a contrasting colour for background material. Experiments reported here indicate that fluorochrome staining with acridine orange can be modified in such a way that a differential staining effect can be achieved.

Techniques for the staining of microorganisms with fluorochromes are in common use for the detection of acid-fast mycobacteria (12). Fluorochrome staining has been employed for studies of bacterial content of water and soil samples (2, 3, 5, 9, 10). The application of acridine orange staining for the detection of malarial parasites in blood smears has been reported (4, 6). Acridine orange is usually preferred in microbiological work. Its use has been hampered, however, by a green-

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Received 4 ii.77 Accepted 4 ii 77

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to-red variation in the staining pattern (1 7 8 11) a variation presumed to correlate with viability (2, 9) We have studied some para meters in order to define optimal conditions for the staining of bacteria and tissue components in dried smears on glass slides. The main objective was to find conditions where all bacteria gave uniform staining also pos sibly of another colour than the background tissue material Such differential fluorescence was achieved when a low pH in the buffer was used resulting in an orange staining of bacteria in contrast to green to-yellow stain ing of human cells and tissue debris in the sample.

MATERIALS AND METHODS

Microorganisms Eighty-one strains of Gram positive and Gram negative bacterial species were obtained from routine cultures. The bacterial species were as follows *Staphylococcus aureus* (13 strains) *S epidermidis* (4 strains) *S saprophyticus* (4 strains) group A, B, and C streptococcus (1? strains) enterococcus (7 strains) diphtheroid ba cillus (1 strain) *Neisseria meningitidis* (1 strain) *N gonorrhoeae* (7 strains) *Escherichia coli* (11 strains) *Klebsiella pneumoniae* (10 strains) *Proteus mirabilis P morganii* and *P vulgaris* (14 strains) *Pseudomonas aeruginosa* (1 strain)

Clinical specimens. Duplicate smears of urethral secretions from male patients with suspected gonococcal infection were obtained from the Depart ment of Dermatology Lund. Smears of tracheal aspirates from patients with artificial respiration were made from samples sent for routine microbiological examination. These samples contained in flammatory and phagocytic cells in various stages of degradation. Thin smears of blood and buccal scrapings from an apparently healthy male volun teer were used to evaluate the staining of less ac tivated inflammatory cells. Very viscous samples were homogenized by adding 5 per cent acetyl cystein (Mukomyst®) followed by centrifugation and suspension in phosphate buffered saline (PBSA 0 12 M naCl 0 03 M phosphate pH 7.3 0 02 per cent sodium azide) No untoward effects of this treatment on the staining results were seen.

Staining solutions Acridine orange (E Merck A.G Darmstadt, No. 1333) was dissolved in 0 15 M buffers ranging from pH 3 5 to pH 9 0 Acetate buffers covered pH values from 3 5 to 5.5 phosphate buffers pH 6 0 to 8 0 and Tris-HCl pH 8.5 and 9 0 Staining was performed in troughs, each taking 8 slides in 50–80 ml of staining solution. The slides were then washed with tap water and dried

Microscopical examination A Leitz Ortholux II microscope equipped with Ploemopak 2.2, filter combination K, for incident light fluorescence was used throughout the studies. Microphotographs were taken with Nikkormat EL and FT2 cameras fitted on a Leitz shutter attachment on the microscope, using Kodak Plus-X and Panatomic-X black and white films and Kodachrome 64 film for colour slides.

RESULTS

Influence of pH on Acridine Orange Staining Patterns

The effect of the hydrogen concentration during staining was studied on isolated bac teria and on inflammatory cells from various sources. Smears of *Staphylococcus aureus* and *Escherichia coli* were fixed with methanol for two minutes and stained for five minutes with 20 mg per litre acridine orange in buffers ranging from pH 3.5 to 9 0 with 0.5 pH unit intervals. Both strains showed intense orange fluorescence throughout the whole range of pH values, with very little variation in colour and intensity Smears of normal blood, mucopurulent tracheal exudate from patients with artificial respiration and urethral secretions from patients with culture positive Gc infec tions were fixed in methanol and stained with 20 mg per litre acridine orange in buffers of different pH and examined in the fluor escence microscope. The staining pattern of polymorphonuclear cells (PMNs) showed clear correlation with the pH of the staining buffer (Table 1) At low pH only the nucleus of blood PMNs was visible with faint green fluorescence At higher pH values, the nucleus stained more strongly with green-to-yellow fluorescence and a green cytoplasm (Table 1) Polymorphonuclear cells from exudates in general gave stronger staining apparently related to their degree of activation (Fig 1 Table 1) At low pH the cells were still green, whereas at neutral pH or higher the orange staining of PMNs made the detection of bacilli in the smears more difficult (Fig 1A and 1B) Free nuclei from disintegrated PMNs showed a reddish staining also at low pH Mononuclear cells were similar to PMNs in their staining pattern. Lymphocyte nuclei

TABLE 1 *Acridine Orange Staining Patterns of Polymorphon lear Leukocyte from Peripheral Blood and from Go-podli e Ureth al Secretions at I arious pH V alues*

pH	Blood PMN		Urethr PNN	
	Nucleus	*Cytoplasm*	*N cleus*	*Cyt pl sm*
3 0–5 0	Faint green	Unstained	Green	Faint green
5.5–8 0	Green	Faint green	Yellow	Green
8 5–9 0	Yellow	Green	Orange	Yellow

Bacteria in secretions and from cultures showed orange fluorescence throughout the pH range

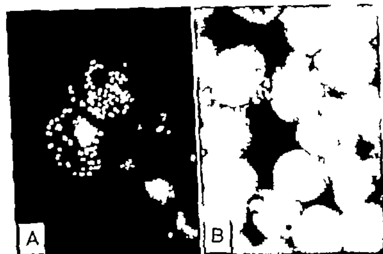


Fig 1 Acridine orange staining (25 mg per l) of human urethral secretions containing *N gonorrhoeae* When stained at pH 4 0, as shown in Fig 1A, the cocci stand out with an orange colour against green background At pH 8 5, Fig 1B, also the PMN the smear stain yellow to orange. × 1,500

ga orange fluorescence already at low pH alues Erythrocytes in blood smears followed the same staining pattern as the background material, e.g. no to faint green fluorescence at low pH to clear green at high pH

Bacteria present *in tracheal* exudates and urethral secretions were also examined with regard to their staining patterns. Irrespective of the pH of the staining buffer the bacteria alway showed strong orange fluorescence (Fig 1) They were however more easily detectable in smears stained at low pH which had the least sta ning effect on the surrounding cells and background material (Fig 3A) Intracellular bacteria including bacteria over laying the nucleus were easily detected, thus indicating the potential advantages of acridine orange staining at low pH. Fading of fluorescence was not so marked with orange staining of the bacteria, whereas the green background faded rapidly

Standardization of Staining Conditions

The influence of various parameters on the staining of bacteria was examined in order to ensure reproducible staining. Furthermore the influence of dye concentration on the staining of bacteria was studied. Acridine orange solutions ranging from 2 to 100 *mg* per litre in a

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8 5–9 0	Yellow	Green	Orange	Yellow

Bacteria in secretions and from cultures showed orange fluorescence throughout the pH range

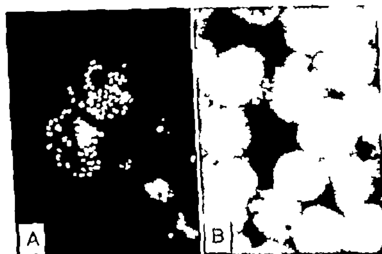


Fig. 1 Acridine orange staining (25 mg per l) of human urethral secretions containing N. gonorrhoeae. When stained at pH 4 0, as shown in Fig. 1A, the cocci stand out with an orange colour against a green background. At pH 8.5 Fig. 1B, also the PMN in the smear stain yellow to orange × 1,500.

gave orange fluorescence already at low pH values. Erythrocytes in blood smears followed the same staining pattern as the background material, e.g. no to faint green fluorescence at low pH to clear green at high pH.

Bacteria present in tracheal exudates and urethral secretions were also examined with regard to their staining patterns. Irrespective of the pH of the staining buffer the bacteria always showed strong orange fluorescence (Fig. 1). They were however more easily detectable in smears stained at low pH which had the least staining effect on the surrounding cells and background material (Fig. 3A). Intracellular bacteria including bacteria overlaying the nucleus were easily detected, thus indicating the potential advantages of acridine orange staining at low pH. Fading of fluorescence was not so marked with orange staining of the bacteria, whereas the green background faded rapidly.

Standardization of Staining Conditions

The influence of various parameters on the staining of bacteria was examined in order to ensure reproducible staining. Furthermore the influence of dye concentration on the staining of bacteria was studied. Acridine orange solutions ranging from 2 to 100 mg per litre in a

neutral buffer (PBSA) were used for 5 minute staining of *S aureus* and *E coli* From 100 *mg down to 20 mg acridine orange per litre* gave strong orange fluorescence of the bacteria. Ten mg per litre and less of acridine orange gave only weak green-to-yellow staining Concentrations between 25 and 50 mg per litre seemed suitable for bacterial microscopy By varying the staining time between half a minute and 10 minutes with this concentration of acridine orange full uptake of dye developed already after 1 minute with no further changes up to the 10 minutes tested. A staining time of 2 minutes was selected as optimal for *routine staining which* was always performed in troughs to ensure proper buffering Various methods for fixation of *S aureus* and *E coli* smears were also tested Methanol chloroform, ethyl ether ethanol 1 1 and 10 *per cent formaldehyde were used as fixatives* for 10 minutes and compared with unfixed air-dried smears. No differences were seen between any of the procedures and the control. Similarly varying the time for methanol fixation between *1 and 30 minutes* did *not* change the staining properties of the bacteria Methanol fixation for 2 minutes is used routinely for direct smears of human material, and was therefore retained in further experiments.

Acridine Orange Staining of Various Isolated Microorganisms

The standard staining conditions selected were tested further on 81 bacterial strains from routine cultures. The bacterial strains represented 15 different species covering both Gram positive and Gram negative cocci and bacilli The staining method consisted of methanol fixation for 2 minutes followed by brief washing in tap water and then immersion in *acridine orange, 25 mg per litre* acetate buffer 0 15 M pH 4 0 for 2 minutes. The slides were washed in tap water and dried and then examined in the fluorescence microscope with immersion oil applied to the smears. In *all cases, the bacteria showed strong* orange fluorescence after staining with acridine orange. On a few slides there were occasional areas with about 10 per cent yellow-stained bacteria By increasing the concentration of *acridine orange to 50 mg per litre* this percentage decreased. This concentration should thus be preferred for routine use

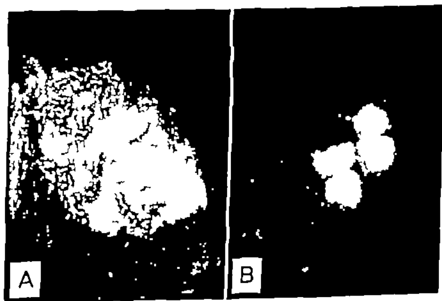


Fig 2 Acridine orange staining at low pH Fig 2A shows an pithelial cell from buccal scraping with bacteria adhering × *500 Fig. 2B shows a smear of a* cerebrospinal fluid specimen from a patient with pneumococcal meningitis. × 800 Bacteria in both 2A and 2B tain orange against a green background of human cells.

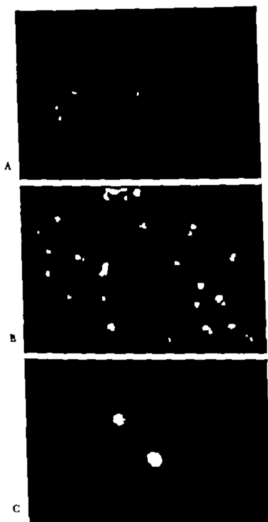


Fig. 3 Acridine orange staining of clinical specimens using an acidic staining buffer pH 3.0. Fig 3A, Exudate from male urethra showing *Neisseria gonorrhoeae* Fig. 3B Cerebrospinal fluid specimen from case of meningococcal meningitis Fig. 3C, Cerebrospinal fluid specimen from a patient with meningitis caused by *Pseudomonas aeruginosa* A × 1000, B and C × 500

Acridine Orange Staining of Clinical Specimens

The standardized acridine orange staining was also applied to smears of clinical specimens Buccal scrapings containing epithelial cells, leucocytes and bacteria were examined. The orange fluorescence of the bacteria contrasted well with the surrounding human cells, as shown in Fig 3 A. The staining pattern at low pH described above for leucocytes was also true for these specimens. The epithelial cells showed faint green cytoplasmic fluorescence and a nucleus with a green-to-yellow colour The bacteria were easily detected also when they adhered to the cells.

Smears of cerebrospinal fluid samples from patients with suspected bacterial meningitis were stained with acridine orange at pH 4.0 in addition to routine Gram and methylene blue staining (Fig 2 B, 3 B, 3 C) Results of a small series indicated that acridine orange was superior to the other two methods for detecting small numbers of bacteria, for instance when only a few meningococci were present on each smear (Fig 3 B) Acridine orange therefore supplemented the Gram and methylene blue staining methods.

The staining method was also tested for use in venerological practice by comparing it with methylene blue staining of urethral secretions from cases of suspected gonococcal infections (Fig 3 A) In 16 out of 17 cases the two staining methods were in agreement, 4 being Gc-negative and 12 Gc-positive. In one case the acridine orange staining was positive methylene blue staining negative and the culture showed growth of *N. gonorrhoeae* Re-examination of the methylene blue stained smear revealed occasional diplococci.

DISCUSSION

Acid fast staining methods using fluorochromes seem to be superior to Ziehl-Neelsen staining techniques for detecting mycobacteria in clinical specimens (12) This is attributed to the excellent contrast obtained between the stained bacteria and the dark background, thus making the examination of such slides both easy and thorough. Direct fluorochrome staining of other non-acid-fast bacteria will present difficulties because of the uptake of dye by other cells as well as by the back

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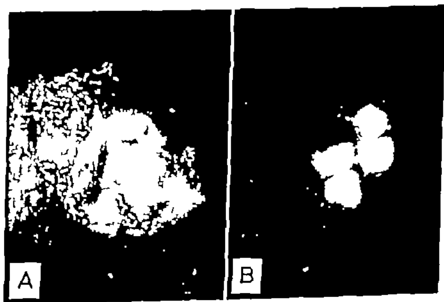


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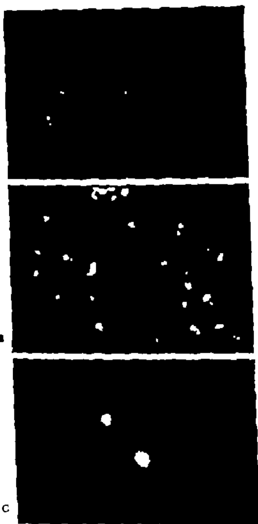


Fig. 3. Acridine orange staining of clinical specimens using an acidic staining buffer pH 3.0. Fig. 3A, Exudate from male urethra showing *Neisseria gonorrhoeae*. Fig. 3B, Cerebrospinal fluid specimen from a case of meningococcal meningitis. Fig. 3C, Cerebrospinal fluid specimen from a patient with meningitis caused by *Pseudomonas aeruginosa*. A × 1000, B and C × 500.

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ground material in smears. We present here a staining technique for the detection of bacteria in clinical specimens which gives a differential coloration of these structures. The method utilizes the pH dependence of acridine orange staining patterns of human cells. Our results have indicated that both human epithelial and inflammatory cells show green-to-yellow fluorescence of the nucleus and no or green fluorescence of the cytoplasm at low pH. Since bacteria give strong orange fluorescence at these pH values the conditions for a differential staining method are obtained. A pH dependence for acridine orange has been described by *Strugger* (7) for plant cells.

Differences were noted in acridine orange staining of polymorphonuclear cells from various sources. Blood PMNs were stained green throughout the whole pH range whereas PMNs from inflammatory exudates gave yellow-to-orange fluorescence already at neutral pH. In some cases of tracheal exudates the PMNs were almost red already at acid pH in the same way as free nuclei. The tendency to give a more orange staining seemed to correlate with the degree of activation and subsequent autolysis of PMNs.

Acridine orange staining at low pH of clinical specimens, particularly cerebrospinal fluid samples and urethral secretions, seems to offer a valuable complement to other staining methods. Apart from its simplicity its main advantage is the marked differential staining effect with orange colouring of bacteria in contrast to green-to-yellow staining of human cells and background material.

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Acta path. microbiol. scand. Sect. B, 85 255-261 1977

THE LYTIC EFFECT OF LYSOLECITHIN ON ACHOLEPLASMAS AND MYCOPLASMAS

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Soltesz, L. V & Mårdh, P.-A. The lytic effect of lysolecithin on acholeplasmas and mycoplasmas Acta path. microbiol. scand. Sect. B 85 255-261 1977

Lysolecithin exhibits a lytic activity on acholeplasmas and mycoplasmas. The acholeplasma studied, ie. *Acholeplasma laidlawii* A and B, were found less susceptible than were the mycoplasmas, ie. *Mycoplasma gallisepticum* and *M pneumoniae* The sensitivity to lysis was found to differ according to species, growth temperature and number of organisms used. Variations in age of the population and the concentration of bivalent ions in the test medium had but little influence. The greatest lytic activity of lysolecithin was found at the optimal growth temperature and decreased with lowering of the temperature. This lytic activity was inversely proportional to the density of the cell suspensions used. The possible mechanisms of the lytic effect of lysolecithin is discussed.

Key words Lysolecithin lytic effect acholeplasmas mycoplasmas

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Received 3.ii.77 Accepted 3.ii.77

Acholeplasmas and mycoplasmas, like L-phase variants of bacteria, lack a rigid cell wall. Its absence explains many of the characteristics of these organisms, such as their morphological instability osmotic sensitivity resistance to penicillin and their susceptibility to detergents, such as saponin and digitonin (14)

Lysolecithin is lethal to acholeplasmas and mycoplasmas (8) *Mårdh & Taylor Robinson* (10) showed that acholeplasmas are less susceptible to lysolecithin than mycoplasmas. L-phase variants of bacteria were found to be at least 100-fold more susceptible to lysolecithin than were their corresponding parent strains (11) Lysolecithin can alter the permeability of cell membranes (9) Nothing is known of the cell membrane receptor for lysolecithin, though it seems likely that the substance interacts primarily with membrane lipids.

Cholesterol affects the packing of fatty acids in the membrane resulting in changes in its permeability which can interfere with the cell's integrity (3 4 5) Lysolecithin may interact with cholesterol, rendering it unavailable for interaction with other membrane lipids. The acholeplasmalytic and mycoplasmalytic effect of lysolecithin is influenced by the amount of cholesterol present in the culture medium (10)

The present report describes a study of the lytic effect of lysolecithin on *Acholeplasma laidlawii* A, *A laidlawii* B, *Mycoplasma pneumoniae* and *M gallisepticum*. The variation in the lytic efficiency according to the age of the organisms and the growth temperature, and the cell density in relation to the concentration of lysolecithin were all assessed, as

ground material in smears. We present here a staining technique for the detection of bacteria in clinical specimens which gives a differential coloration of these structures. The method utilizes the pH dependence of acridine orange staining patterns of human cells. Our results have indicated that both human epithelial and inflammatory cells show green to-yellow fluorescence of the nucleus and no or green fluorescence of the cytoplasm at low pH. Since bacteria give strong orange fluorescence at these pH values, the conditions for a differential staining method are obtained A pH dependence for acridine orange has been described by *Strugger* (7) for plant cells.

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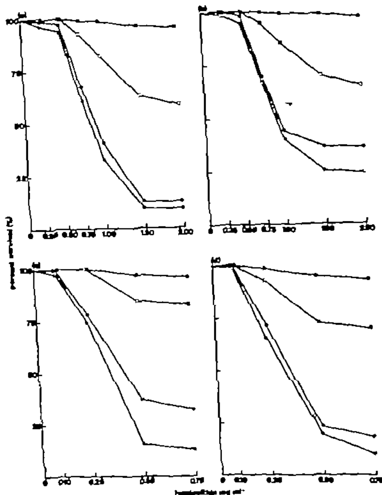


Fig. 2. Influence of temperature on the lytic effect of lysolecithin on *A. laidlawii* A (a), *A. laidlawii* B (b), *M. gallisepticum* (c) and *M. pneumoniae* (d). 100 per cent survival equal to 10^3 cells/ml. ○—○ 37° C, ●—● 25° C, □—□, 15° C; and ■—■ 4° C.

g for 15 minutes and washed twice in 0.25 M saline before testing. The organisms were then suspended in 4 ml of the saline to which lysolecithin at the various concentrations had been added. When not otherwise indicated, the mixtures were incubated at 37° C for 15 minutes and the degree of lysis was determined by measuring the changes in optical density at 500 nm using a Unicam spectrophotometer (Cambridge, England) model SP 800A.

Various cell densities and organisms of varying age were used, as indicated below. Organisms grown in the presence of cholesterol for various periods of time were also tested for their susceptibility to lysolecithin. In these experiments, 750 µg per ml lysolecithin was added to broth cultures containing 1×10^8 cells ml^{-1}. The number of organisms was determined spectrophotometrically and by counting the number of colony-forming units (c.f.u.) under a stereomicroscope. The cholesterol content was 5 per cent of the dry weight of the organisms as determined when the culture contained 1×10^8 cells ml^{-1}.

The results are given as the difference, expressed in per cent, between the optical density of the cultures which did and did not contain lysolecithin and which had been treated under identical conditions.

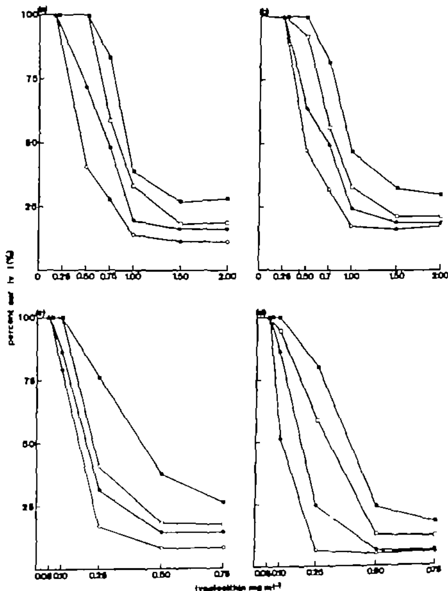


Fig 1 The lytic effect of lysolecithin on *A laidlawii A* (a) *A laidlawii* B (b) *M gallisepticum* (c) and *M pneumoniae* (d) as a function of cell density ○——○ 10^2 ●——● 10^4 □——□ 10^6 and ■——■ 10^8 cells/ml.

was the inhibitory effect of cholesterol on the lytic process studied. The possible mechanism of the lytic effect of lysolecithin is discussed

MATERIALS AND METHODS

Organisms A laidlawii A (PG 8) and *A laidlawii* B (PG 9) were obtained from professor *E. A Freundt* Aarhu Denmark while *M gallisepticum* (PG 31) and *M pneumoniae* (Mac strain) came from Dr *D Taylor Robinson* CRC, Harrow England.

Growth medium The growth and test medium consisted of Bacto-PPLO broth (Difco) containing 20 per cent (v/v) unheated horse serum, 10 per cent (v/v) of a 25 per cent solution (w/v) of Bacto-Yeast extract (Difco) 0 1 per cent (w/v) glucose 0 05 per cent (w/v) thallium acetate and 1,000 units of penicillin G ml^{-1} The pH was adjusted to 7 8

Chemicals Lysolecithin, prepared from egg lecithin, was obtained from Sigma Ltd., Saint Louis, Missouri, USA, and cholesterol from Kebo AB, Stockholm, Sweden. Lysolecithin was dispersed into the medium direct or in 0 25 M saline while cholesterol was dispersed as described by *Edward & Fitzgerald* (7)

Test conditions. The organisms were harvested from the broth medium by centrifugation at 12,500

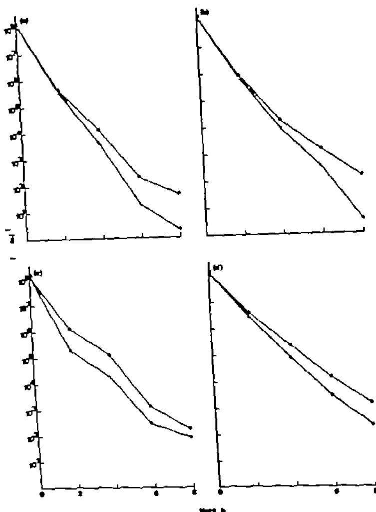


Fig. 5 The influence of cholesterol on the susceptibility of *M. pneumoniae* ( ), *M. gallisepticum* (b), *A. laidlawii* A (c) and *A. laidlawii* B (d) to lysolecithin. Cholesterol not added (○—○), cholesterol added (●—●)

Influence of Cholesterol on Lytic Capacity of Lysolecithin

Cholesterol at a concentration of 730 μg/ml was added to the cultures after 2, 4, 6, and 8 hours of incubation. The broth culture tested contained 10^8 organisms/ml when cholesterol was added. Non-cholesterol-containing cultures, treated in the same way were used as controls (Fig. 5). After the addition of cholesterol, the organisms became more resistant to lysolecithin. This effect was more pronounced for *A. laidlawii* B than for *A. laidlawii* A.

DISCUSSION

The cidal effect of lysolecithin on organisms of the family *Mycoplasmataceae* is ascribable to cell lysis (8). The susceptibility of *M. pneumoniae*, *M. gallisepticum*, *A. laidlawii* A and *A. laidlawii* B to lysolecithin was found to decrease in the order given.

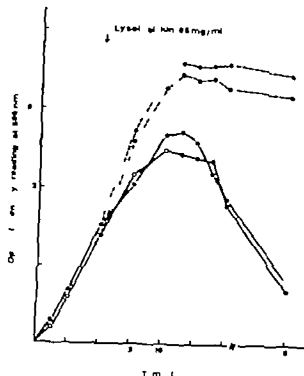


Fig 3 Decrease in cell density after administration of 0 5 mg/ml of lysolecithin to an exponentially growing culture of *A laidlawii* A (●——●) and *A laidlawii* B (○——○) As comparison the cell density in non lysolecithin-containing cultures of *A laidlawii* A (●——●) and *A laidlawii* B (○——○) are shown

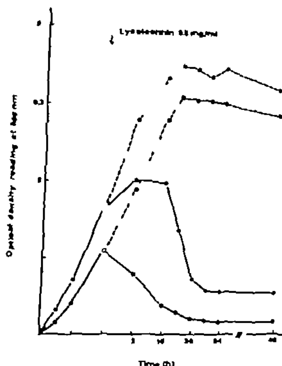


Fig 4 Decrease in cell density after addition of 0.5 mg/ml of lysolecithin to an exponentially growing culture of *M gallisepticum* (●——● and *M pneumoniae* (○——○) As comparison, the cell density in non-lysolecithin-containing cultures of *M gallisepticum* (●——●) and *M pneumoniae* (○——○) are shown.

RESULTS

Lytic Effect of Lysolecithin on Acholeplasmas and Mycoplasmas

Cell density The lytic effect of lysolecithin, in concentrations of 100 to 2 000 μg ml⁻¹ on *A laidlawii* A at various cell densities is shown in Fig 1 a. The corresponding effects of lysolecithin on *A laidlawii* B (Fig 1 b) *M gallisepticum* (Fig 1 c) and *M pneumoniae* (Fig 1 d) were also studied In these experiments the concentrations of lysolecithin was varied between 50 and 800 μg ml⁻¹

In the test with the two species of *Acholeplasma* the maximum lytic effect occurred when using 10^3 organisms ml⁻ and 125 μg per ml lysolecithin (Figs. 1 a and 1 B) When testing *M gallisepticum* and *M pneumoniae* the same effect was observed when using the same number of organisms, but with 100 and 750 μg per ml lysolecithin, respectively (Figs. 1 c and 1 d)

Temperature The influence of temperature was studied on the lytic effect of lysolecithin on the four species tested. The lytic effect on all species was at its maximum at 37° C (Fig 2 a–d) and diminished with falling temperatures.

Difference in Susceptibility to Lysolecithin among Species Studied

Lysolecithin was added to cultures after 8 hours incubation. The optical density of the cultures to which lysolecithin had been added was determined at intervals of 2 hours, and aliquots of the suspensions were plated on solid medium in order to determine the number of c.f u. ml⁻¹ The susceptibility to lysolecithin of the two species of *Mycoplasma* was greater than that of *Acholeplasma*. *M pneumoniae* was the most and *A laidlawii* B the least susceptible (Figs 3 and 4)

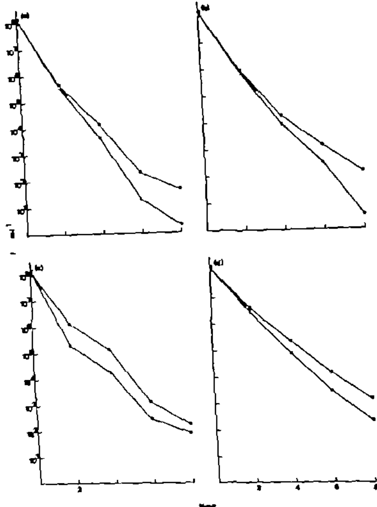


Fig. 5. The influence of cholesterol on the susceptibility of *M. pneumoniae* (a), *M. gallisepticum* (b), *A. laidlawii* A (c) and *A. laidlawii* B (d) to lysolecithin. Cholesterol not added (O——O); cholesterol added (●——●).

Influence of Cholesterol on Lytic Capacity of Lysolecithin

Cholesterol at a concentration of 750 μg/ml was added to the cultures after 2, 4, 6, and 8 hours of incubation. The broth culture tested contained 10^9 organisms/ml when cholesterol was added. Non-cholesterol-containing cultures, treated in the same way were used as controls (Fig. 5). After the addition of cholesterol, the organisms became more resistant to lysolecithin. This effect was more pronounced for *A. laidlawii* B than for *A. laidlawii* A.

DISCUSSION

The cidal effect of lysolecithin on organisms of the family *Mycoplasmataceae* is ascribable to cell lysis (8). The susceptibility of *M. pneumoniae*, *M. gallisepticum*, *A. laidlawii* A and *A. laidlawii* B to lysolecithin was found to decrease in the order given.

17*

Acholeplasmas in contrast to mycoplasmas, do not require sterols for growth. However acholeplasmas are able to incorporate cholesterol when present in the growth medium, which fact renders these organisms suitable for studies on the role of cholesterol in biological membranes.

The non-electrolyte permeability of the cytoplasmic membrane of acholeplasmas is reduced by the incorporation of cholesterol (13) Cholesterol reduces the molecular motion of the hydrocarbon chains of unsaturated phospholipids resulting in their closer packing It interacts preferentially with fatty acid chains in the liquid-crystalline state (6) Changes in the fatty acid composition of the membrane lipids of acholeplasmas may compensate for the lack of cholesterol (15)

Lipid depletion of mycoplasma cell membranes greatly reduces the cholesterol uptake, while removal of most of the membrane proteins does not significantly affect the cholesterol content (13) This indicates that the major portion of cholesterol bound to mycoplasma membranes is incorporated into the lipid domains of the membranes.

Lysolecithin interacts with lipid components of cell membranes resulting in an integration of lysolecithin into the membranes, followed by a gradual aggregation of lipid lysolecithin complexes of the membrane. This aggregation is dependent on the thermal motility of the fatty acid components of the membrane and takes place more rapidly at higher temperatures. The exterior parts of these aggregates are hydrophobic and interact with the surrounding membrane lipids. When the aggregates become sufficiently large they may form a ring around a hydrophil pore. The permeability barrier is then broken thus finally leading to cell lysis. When cholesterol is present the cholesterol lysolecithin binding is stronger than the cholesterol membrane lipid binding If there is a surplus of cholesterol, it can react with the lipid component of the cell membrane (6) This may explain the difference in susceptibility among the species of *Acholeplasma* and *Mycoplasma* studied, since it is known that the amount of cholesterol in the cell membranes of these species varies (1)

A low temperature can reduce the fluidity of the membrane lipids and thereby alter the mechanical properties of the membrane. The ability of lysolecithin to interact with the membrane is also temperature-dependent. The damage to the cell integrity of acholeplasmas and mycoplasmas by lysolecithin was found negligible below 10 C. Lysolecithin-induced lysis of these organisms requires a certain state of fluidity of the membrane lipids.

Rearrangements in the liquid-crystalline state of the fatty acids may alter the permeability properties of a cell membrane (14) A rearrangement of the fatty acids in the lipid domain of the cell membrane might also explain the slight difference in susceptibility to lysis by lysolecithin, which was observed when using organisms of differing ages.

The presence of lysophospholipase in the cell mebrane of *A laidlawii* B (16) might, at least partially explain why these organisms were those most resistant to lysolecithin among the species studied The presence of similar lysophospholipase activity in *A laidlawii* A and the two species of *Mycoplasma* studied has not been so far reported (2)

One advantage of using lysolecithin for the lysis of acholeplasma and mycoplasma cells, compared with osmotic lysis, is that the lytic effect of lysolecithin is relatively unaffected by the age of the organisms. Lysolecithin may also be useful for the isolation of cell membranes of slow-growing mycoplasmas. When using this compound, one is less restricted to a fixed harvesting time than when using osmotic lysis. Species of *Mycoplasma* such as *M gallisepticum* which are more resistant to osmotic lysis than are other mycoplasma species, can also be lysed easily with lysolecithin.

Structural, metabolic and energetic factors may be involved in the sequences of events leading to cell lysis by lysolecithin. Which of these factors plays the major role may vary according to the growth conditions and the physiological status of the cell

To conclude – the cell integrity of acholeplasmas and mycoplasmas is impaired when

lysolecithin interacts with the lipid components of cell membranes. Lysolecithin forms aggregates with lipids which influence the permeability properties of the cell membrane resulting in lysis of the cells.

This study was supported by grant No. 16X-4509 from the *Swedish Medical Research Council*.

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Acta path microbiol. scand. Sect. B 85 262–270 1977

CHEMICAL COMPOSITION, SEROLOGICAL REACTIVITY AND ENDOTOXICITY OF LIPOPOLYSACCHARIDES EXTRACTED IN DIFFERENT WAYS FROM *BACTEROIDES FRAGILIS*, *BACTEROIDES MELANINOGENICUS* AND *BACTEROIDES ORALIS*

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Hofstad, T Sveen, K. & Dahlén, G Chemical composition, serological reactivity and endotoxicity of lipopolysaccharides extracted in different ways from *Bacteroides fragilis Bacteroides melaninogenicus* and *Bacteroides oralis* Acta path. microbiol. scand. Sect. B, 85 262–270, 1977

Lipopolysaccharides (LPS) extracted from strains of *Bacteroides fragilis Bacteroides melaninogenicus* and *Bacteroides oralis* with phenol-water trichloroacetic acid, EDTA or liquid phenol-chloroform petroleum ether (PCP) and isolated by ultracentrifugation, varied considerably in their quantitative chemical composition. Negligible yields of LPS were obtained by PCP extraction. All preparations were more or less serologically active. All methods (except PCP) extracted the same O-antigenic determinants from *B fragilis*. Endotoxic activity as measured by primary skin inflammations in rabbits, was low but was present in all preparations. Proteins (and/or lipoproteins) co-precipitated with LPS in the ultracentrifuge.

Key words *Bacteroides fragilis B melaninogenicus B oralis* lipopolysaccharides extraction.

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Received 9 ii 77 Accepted 3 iii.77

Several methods have been used for extraction of bacterial lipopolysaccharides (LPS) (22) The extensively used phenol-water (P/W) method extracts the LPS nucleic acids and, if present in the cells, other polysaccharides such as capsular antigens and glucans. Some other extraction methods, for instance extraction with trichloroacetic acid (TCA) and aqueous ether have in common that phospholipid lipoprotein and protein may be co-extracted often in the form of a complex. In recent years, LPS have been isolated from fragments of outer cell membranes removed by treatment of whole cells with ethylenediamine tetraacetic acid

(EDTA) (24 30) Extraction with liquid phenol-chloroform-petroleum ether (PCP) (6) has been employed for isolation of LPS from rough mutants.

For some years we have used P/W extraction followed by ultracentrifugation of the water phase and enzymatic treatment for isolation of LPS from anaerobic bacteria (10) Problems have been encountered in preparing LPS from strains of *Bacteroides fragilis Bacteroides melaninogenicus* and *Bacteroides oralis*. Preparation of LPS from *B. melaninogenicus* and *B oralis* has regularly had a high glucose content, indicating contamination with glucans (7 3) This has often been true also for *B. fragilis* LPS (11) The yield of LPS has been relatively poor and sometimes the preparations have been contaminated with proteins. Furthermore, the endotoxic activity of the preparations has been low (9 13 28) Extraction of whole or crushed cells of *B. fragilis B melaninogenicus* and *B oralis* with P/W has, therefore been compared with TCA and PCP extractions, and gentle treatment with EDTA with respect to chemical composition, serological reactivity and endotoxic activity of materials isolated from the extracts by ultracentrifugation. The preparations were also examined by electron microscopy

MATERIALS AND METHODS

Organisms

The microorganisms used were *Bacteroides fragilis ss fragilis* strain NCTC 9343 *Bacteroides melaninogenicus ss. intermedius* strain B10 (7) and *Bacteroides oralis* Bact MC3 (3)

Cultural Conditions

Cultures of B10 were grown in 500 ml screw-cap bottles, filled to the top with the following medium (g/l) Tryptone (Oxoid) 15.0; NaCl 5 0 KH_2PO_4 1.5 $Na_2HPO_4 \cdot 2H_2O$ 3 5 $(NH_4)_2SO_4$ 0.5; glucose 2.5; yeast extract (Oxoid) 3 0 L-cysteine HCl 1.0 haemin 0 016 menadione 0 0006 human plasma 50 ml/l, pH 7.0. NCTC 9343 was grown in chemostat (BioTec FT 104, BioTec AB, Stockholm, Sweden) at pH 7.0 and at a dilution rate of 0.7 h⁻¹. The medium was the same, except that human plasma was omitted and 0.5 per cent glucose was used as energy source and growth-limiting factor Bact MC3 was grown in a stirred fermentor at pH 7.2. The medium used was Brain Heart Infusion (Difco 0037-01) supplemented with L-cysteine HCl 0.05 per cent yeast extract (Oxoid L21) 5 per cent glucose 1 per cent liver extract 5 per cent menadione 0.1 per cent and haemin 0 1 per cent.

Preparation of LPS

Extraction with phenol-water (P/W) Suspensions in distilled water of 20 mg freeze-dried, washed cells per ml or 100 mg packed, washed cells per ml, were homogenized with equal volumes of 90 per cent phenol (29) for 15 min at room temperature (20–22 C) After centrifugation for 30 min at 2500 × *g* the water phase was pipetted off and dialyzed against tap water for three days, and the volume reduced by evaporation. LPS were purified from the evaporated water phase by ultracentrifugation (100,000 × *g* for 90 min) and treatment with deoxyribonuclease and ribonuclease (15)

LPS were also prepared from crude cell walls (19) Washed and frozen cells were crushed by five passages through the X-press (AB BIOS, Nacka, Sweden) (4) extracted overnight at 4 C with 0.05 M phosphate buffer pH 7 4 and defatted at –25 C with ethanol-ether (2 1) After washing in acetone, the dried cells were extracted and purified as described above.

Extraction with trichloroacetic acid (TCA) (1) Freezedried, washed organisms were suspended in ice-cold 5 per cent TCA to 200 mg per ml and extracted under continuous stirring for 3 h at 4 C. Thereafter the suspension was heated to about 20 C and centrifuged at the same temperature for 15 min at 10,000 × *g* The supernatant fluid was collected and the precipitate re-extracted with cold TCA. The pooled supernatants were dialyzed against tap water for two days, the volume reduced by evaporation, and LPS purified by ultracentrifugation and treatment with deoxyribonuclease and ribonuclease.

Extraction with 90 per cent liquid phenol-chloroform-petroleum ether (b p. 40–60° C) 2.5.8 (PCP) was performed exactly as described by *Galanos et al.* (6)

Extraction with ethylenediamine tetraacetic acid (EDTA) Suspensions of 1.0 g washed, freeze-dried cells in 10 ml of phosphate-buffered saline, pH 7 4 were made. EDTA (2.0 mM pH 7.3) was added to give a final concentration of EDTA in the suspensions of 0 1 mM (2) The suspensions were homogenized using magnetic stirrer for 5 min at room temperature (20–22 C) and centrifuged for 20 min at 8000 × *g* at 4 C. The supernatant fluid was pipetted off and LPS purified by ultracentrifugation.

TABLE 1 *Composition of LPS Extracted in Different Ways from* B. fragilis *NCTC 9343 and Purified by Ultracentrifugation*

Method of extraction	Yield mg	Protein per cent	Fatty acid esters per cent	Phosphorus per cent	Neutral sugars							Amino sugars per cent
					Total per cent	Mol per cent of						
						Glc	Gal	Fuc	Rha	Rib		
P/W dried cells 4 8 g	145	2 9	3.9	0.2	87.2	96.5	1.9	0 8	0.8			3 0
Crude walls, wet cells 48 g	20	17 0	8.3	1 6	35.8	89 1	6 4	2 4	2.1			n.d.
TCA dried cells 5 g	182	6.5	13 0	1.3	45 6	88.9	7.5	1 7	1.9			6 8
EDTA dried cells 2 g	37	61 0	18 8	1.3	5.2	14.2	22.8	23.3	18.2	21.5		1.5
PCP dried cells 5 g	4 8	n d.	n.d.	n.d.	24.2	18.6	56 6	7 4	13 6	3.8		n.d.

n.d. = not done.

TABLE 2. *Serological Activity and Endotoxicity of LPS Extracted in Different Ways from* B. fragilis *NCTC 9343 and Purified by Ultracentrifugation*

Method of extraction	Minimal dose giving		Inhibition of HA c) SRBC sensitized with LPS-					SLD$_{50}$ μg
	Precipitation a) μg/ml	Sensitization of SRBC b) μg/ml	P/W μg	P/W walls μg	TCA μg	EDTA μg	PCP μg	
P/W								
whole cells	25 0	6 25	1.25	1 25	1 25	25.0	n.d.	30 0
crude walls	6.25	1.56	0 62	0.62	1.25	1 25	n.d.	47.5
TCA	12.5	3 12	0 62	0 62	0 62	0 62	n.d.	65 0
EDTA	50 0	25 0	0 62	5.0	2.5	5 0	n.d.	75 0
PCP	n.d.	12.5	>5.0	n.d.	>5.0	2.5	0 62	16 7

a) Lowest concentration of LPS to give positive ring test.
b) Lowest concentration of LPS to give maximal serum titres.
c) 8 agglutinating units of antiserum.
n.d = not done.

TABLE 3. *Inhibitory Activity in the Test Systems for Demonstration of the Antigenic Factors 2, 3 and 5 (11) of LPS Extracted in Different Ways from Bacteroides fragilis NCTC 9343 and Purified by Ultracentrifugation*

Factor	Test system		Minimal inhibiting dose (μg) of LPS 9343			
	Absorbed antiserum a)	Sensitizing antigen b)	P/W whole cells	P/W crude walls	TCA	EDTA
2	Anti-9343 abs. LPS-B55	LPS-E 323	5	5	1.25	5
3	Anti-9343 abs. LPS E 323	LPS-B55	0.62	0.62	2.5	5
5	Anti-9343 abs. LPS B55 + E 323	LPS-9343	1.25	1.25	1.25	2.5

a) 8 agglutinating units of absorbed antiserum.
b) Sensitizing dose of LPS (P/W; whole cells) was 4 times the amount necessary to give maximal serum titre.

Chemical Analyses

Protein was measured by the Folin-Ciocalteu phenol method (21) using bovine serum albumin as standard. The method of *Fiske & Subbarow* (5) as modified by *Youngburg & Youngburg* (31) was used for determination of phosphorus. Fatty acid esters were measured as tripalmitin by the hydroxamic acid method of *Snyder & Stephens* (27). Amino sugars were estimated as glucosamine (23) and samples were hydrolysed with 3 N HCl for 6 h.

Gas Liquid Chromatography (GLC) of Neutral Sugars

Samples hydrolysed with 0.1 N HCl at 100° C for 48 h were neutralized with Amberlite IRA 410 HCO form and the aldoses converted to alditol acetates as described by *Sawardeker et al.* (25). GLC was run in a Perkin-Elmer 900 Gas Chromatograph with a flame ionization detector and fitted with glass column (0.20 × 180 cm) packed with 3 per cent ECNSS-M (w/w) on Gas-Chrom Q, 100/120 mesh (Applied Science Laboratories, State College, Pa., USA). The flow of gas (N) was 30 ml/min and the column and detector temperatures 190 and 260 C, respectively. D-xylose was used as internal standard.

Electron Microscopy

Lyophilized LPS were suspended in distilled water by sonication, stained with 2 per cent uranyl acetate in distilled water at pH 4.5, and prepared as described by *Shand et al.* (26) for microscopy in a Hitachi, Model HU-12 A, electron microscope, at 100 kV.

Primary Skin Inflammation (20)

The LPS preparations were suspended in sterile isotonic saline and sonicated. Serial two-fold dilutions were made in sterile saline and suspensions of LPS (0.2 ml) were injected intradermally in horizontal rows below the median line of the dorsum. Each preparation was tested in 5 rabbits. Reactions measuring 5 × 5 mm or more present 48 h after injection were recorded and used for determination of the skin lesion dose (SLD_{50}).

Serological Methods

Rabbit antisera were raised against whole organisms (8) or LPS (Bact MC3). Sensitization of sheep erythrocytes (SRBC) with NaOH-treated LPS, the indirect haemagglutination (HA) technique, inhibition of haemagglutination, and preparation of test systems for demonstration of O-antigenic specificities have already been described (11). Ring test precipitation was performed as described previously (8).

RESULTS

LPS 9343

Extraction of whole cells with P/W or TCA gave the best yield of LPS (Table 1). The figures for fatty acid esters, phosphorus and amino sugars varied from one preparation to another. The LPS obtained after extraction with P/W and TCA contained considerable amounts of glucose, whereas substantial amounts of ribose were present in the preparations extracted with EDTA. The

TABLE 4 *Serological Activity and Endotoxicity of LPS Extracted in Different Ways from* Bacteroides melaninogenicus *B10 and Purified by Ultracentrifugation*

Method of extraction	Minimal dose giving Precipitation µg/ml	Sensitization of SRBC µg/ml	Inhibition of HA, SRBC sensitized with P/W µg	P/W walls µg	TCA µg	EDTA µg	PCP µg	SLD_{50} µg
P/W								
whole cells	50 0	6 25	0.31	0 62	>5 0	0 62	n.d.	13 8
crude walls	6 25	6.25	0 16	0 16	0 62	>5 0	n.d.	23 8
TCA	12.5	3 12	0 62	>5 0	0 08	0.31	n.d.	37.5
EDTA	25 0	12.5	1 25	0.31	0 62	0.31	n.d	65 0
PCP	n.d	50 0	n.d.	n.d	n.d.	n.d.	>5 0	9 4

Legend see Table 2.

TABLE 5 *Composition of LPS Extracted in Different Ways from* B. oralis *Bact MC3 and Purified by Ultracentrifugation*

Method of extraction	Yield mg	Protein per cent	Fatty acid esters per cent	Neutral sugars Total per cent	Mol per cent of Glc	Gal	Fuc	Rha	Man	Amino sugars per cent
P/W dried cells 14 6 g	89	2.6	5.5	53 6	77 1	6 0	5 7	10 1	1 1	3 6
Crude walls wet cells 29.8 g	36.8	7 4	8 0	9 1	5 6	41.5	20.8	18.9	13 2	5 0
TCA wet cells 14 g	65.8	12.0	4 4	10 4	30 8	21.5	7 7	18.5	21.5	4 2
EDTA wet cells 16.2 g	37 1	52.5	3 2	0 8	17 4	30 4	17 4	21.8	13 0	1 8



Fig. 1 Lyophilized LPS extracted from *B. melaninogenicus* B 10 with TCA (A) and LPS extracted from *B. fragilis* NCTC 9343 with EDTA (B) and TCA (C). Negatively stained with uranylacetate, pH 7.5. Electron micrographs, magnification × 100,000. Horizontal line represents 100 nm

EDTA preparations were rich in protein (and/or lipoprotein)

All preparations were serologically active (Table 2) Examination of the LPS extracted with P/W TCA and EDTA in the test systems for demonstration of the antigenic determinants 2 3 and 5 (11) showed that all determinants were present in all four LPS preparations (Table 2)

The endotoxic activity of the preparations was low (Table 3)

LPS B10

The best yields of LPS were obtained with extraction of whole cells with P/W or TCA but the preparations contained high amounts of glucose. All preparations had a fatty acid content of about 20 per cent Otherwise the quantitative composition varied as described for LPS 9343

The serological activity is shown in Table 4

The preparations extracted with PCP and P/W showed the lowest SLD_{50} (Table 4)

LPS Bact MC3

No LPS were obtained by extraction with PCP The figures for the different constituents varied from one preparation to another and the recovery of the chemical analyses was low (Table 5) All preparations contained mannose. As much as 400 μg/ml of the LPS extracted with TCA and EDTA was needed for sheep cell sensitization and the preparations showed no inhibitory activity in doses up to 5 μg The SLD_{50} varied from 25 0 μg (P/W crushed cells) to 87.5 μg (EDTA)

Electron Microscopy

All preparations contained particles with a trilaminar surface structure (15 28) The particles were doughnut shaped or appeared in the form of discs or rods (Fig 1 A, B C) The diameter of the disc or doughnut shaped particles and the length of the rods varied from 15 to 100 nm. In addition the preparations of LPS B10 contained some filamentous structures (Fig 1 A)

DISCUSSION

The main implication of the present work is that the O antigen and the endotoxic principle of all three *Bacteroides* species can be extracted with P/W TCA and EDTA.

Negligible amounts of LPS were obtained by extraction with PCP This shows that lipid is not a major part of the LPS complex, as also indicated by previous studies (13 14) Most LPS preparations extracted with P/W and TCA were obviously contaminated with glucans. This contamination could be avoided if EDTA was used for extraction, but, as could be expected, substantial amounts of proteins and/or lipoproteins were co-extracted and sedimented in the ultracentrifuge together with the LPS

The varying molar percentages of neutral sugars indicate that varying amounts of macromolecular carbohydrate-containing polymers other than LPS may have been extracted Such macromolecular compounds may be cell surface components (17)

Extraction of whole cells with P/W or TCA seems preferable to EDTA for extraction of precipitating and erythrocyte-sensitizing antigens. The figures for LPS-9343 (Table 3) and LPS B10 (Table 4) suggest that P/W and TCA extracted different amounts of the same antigenic determinants or separate antigens (LPS B10)

As judged by the capacity to produce skin inflammation in rabbits, the endotoxicity of all preparations was low These findings are in keeping with earlier investigations (9 28) Recently Kasper (18) has isolated from a strain of *B fragilis* a lipopolysaccharide which failed to provoke the local Shwartzman reaction in rabbits and was non-lethal for 10-day old chicken embryos. A poor endotoxic activity is thus a characteristic feature of water soluble LPS isolated from *Bacteroides*

Apart from the filamentous structures found in LPS extracted with TCA from *B. melaninogenicus* B10 there were no principal differences in structure or size of the LPS particles extracted by the different methods.

As indicated by the results of the chemical

examinations, other methods than preparative ultracentrifugation must be used for purification of *Bacteroides* LPS for structural studies. For some years we have tried treatment with nucleases, gel filtration, ion exchange and adsorption chromatography for purification of LPS extracted with P/W from *B. fragilis* (12). Another approach may be fractionation of watersoluble or dispersable materials from crushed cells treated with lysozyme and EDTA (16). In the present study low yields of LPS were obtained after treatment of whole cells with EDTA. However, the concentration of EDTA was low and may not have been optimal for extraction of LPS from *Bacteroides* species.

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Acta path. microbiol. scand. Sect. B, 85 271–276 1977

ORIGIN OF INTESTINAL β-GLUCURONIDASE IN GERMFREE MONOCONTAMINATED AND CONVENTIONAL RATS

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Rød, T O & Midtvedt, T Origin of intestinal β-glucuronidase in germfree, monocontaminated and conventional rats. Acta path. microbiol. scand. Sect. B, 85 271–276, 1977

The intestinal β-glucuronidase was studied in germfree, monocontaminated and conventional rats. The greater part of the β-glucuronidase of the caecum and the large intestine of the contaminated animals was of bacterial origin. No bacterial β-glucuronidase was found in the small intestine. Monocontamination with *Escherichia coli* gave activities corresponding to those of the conventional rats, whereas content from the caecum and the large intestine of the rats monocontaminated with *Streptococcus pyogenes* showed an activity approximately 10 per cent of that of the conventional rats.

Key words Intestinal β-glucuronidase origin rats germfree life.

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Received 21.II.77 Accepted 5.iv.77

The study of metabolism in the gastrointestinal tract has emphasized primarily the role of the animal tissue. During the last years, however, increasing interest has been focused on the metabolic activities of the intestinal microorganisms. Hydrolysis of glucuronides is considered to be one of the more important of the gastrointestinal reactions. This might be carried out by animal or bacterial enzymes and may have physiological and pharmacological implications (5 7 23 25) as well as being of significance in intestinal diseases (20)

Bacterial β-glucuronidases (E.C.3.2.1.31) have been shown to act either primarily intracellularly (10) or extracellularly (11) In the latter case, enzyme induction has been found to occur (1 2) Most of the bacterial β-glucuronidases present in the intestinal tract are considered to be produced by anaerobic bacteria (8 12, 23)

Many of the investigations of animal and bacterial β-glucuronidases have been performed by the phenolphthalein glucuronide method. However the advantages of the p-nitro-phenyl method are now indicated in several studies (18, 24 28)

The main object of the present investigation was to study to what extent rat intestinal β-glucuronidase was of animal or bacterial origin, and to see whether monocontamina-

tion could increase significantly the intestinal β glucuronidase activity of the gnotobiotic animal.

MATERIALS AND METHODS

Animals

Germfree, monocontaminated and conventional rats of the CDF strain were reared as described by *Midtvedt & Trippestad* (17) Four rats of each group were studied.

Bacterial Strains

The strain of *Escherichia coli* used for monocontamination was identical with the one described elsewhere (17) The strain of *Streptococcus pyogenes* was kindly provided by Dr *B Hovig* of this institute. The presence of β-glucuronidase in the two strains was assayed by a method described elsewhere (22)

Contamination of Germfree Rats

The strains were transferred to the isolators and the germfree rats were contaminated as described elsewhere (17) The rats were kept monocontaminated for approximately 60 days.

Bacteriological Counting

Samples of the content of the small intestine the caecum, and the large intestine were taken under sterile conditions and suspended 1 10 w/v in Todd Hewitt broth (Oxoid Ltd. London, England) The suspensions were mixed thoroughly using a Whirlimixer (Scientific Industries International Inc. Ltd., England) and then series of tenfold dilutions were made in Todd Hewitt broth. The tubes were incubated aerobically at 37 C for 18 hours and the growth recorded.

Preparation of Homogenates

The rats were killed by exposure to diethylether Intestinal content was squeezed out of the middle segment of the small intestine the caecum and the large intestine. The intestines ere then cut open and the intestinal mucosa scraped off with a stainless steel spatula. The material was immediately frozen and stored at −20 C Subsequent preparations were performed in ice-cold surroundings. The intestinal content, the mucosa and the rest of the intestinal wall were diluted with sterile, redistilled water and homogenized with a Tri R Stir R homogenizer model S 63 C (Tri-R Instruments Inc New York, USA) and subjected to ultrasonic disintegration (MSE Ultrasonic Disintegrator model 60 W MSE Ltd., London, England) for 3 × 1 minute at 1 33 Amperes. The suspensions were centrifuged at 10 000 × g for 10 minutes, and the supernatant fraction was used in the determination of the enzyme activity

Estimation of β-glucuronidase Activity

The pH optimum of β-glucuronidase activity was determined for the intestinal content from the caecum of all rats, and for the caecal mucosa of the germfree rats. Further analyses of the mucosa and the rest of the intestinal wall were performed at pH 4.5 and analyses of the intestinal content at pH 4.5 and 6.5

The buffers contained 0.1 M acetic acid/Na-acetate 0 1 M NaH_2PO_4/Na_2HPO_4 0.2 per cent vol/vol Triton X 100 (E. Merck, Darmstadt, Germany) and 2 mg bovine serum albumin (Sigma) per ml. A solution of 20 mM p-nitrophenyl-β-glucuronide (E. Merck) was used as substrate, giving a final assay concentration of 5mM. The reaction mixture consisted of 2 vol. buffer 1 vol. homogenate and 1 vol substrate solution. Incubations were performed at 37 C for 15–60 minutes. The reaction was terminated and the colour developed by the addition of 2 vol. 1 M K_2CO_3 Blanks were prepared in a similar manner but the K_2CO_3 solution was added before the substrate. The samples were read by spectrophotometer (Spectronic 20 Bausch & Lomb, Rochester New York, U.S.A.) at 420 nm and compared with a standard curve obtained from a solution of p-nitrophenol (Sigma)

Protein was determined according to *Lowry et al.* (15) Working standards of bovine serum albumin were used throughout. The β-glucuronidase activity is expressed as μmoles of p-nitrophenol liberated/60 minutes per g of protein.

RESULTS

The optimal pH of β-glucuronidase activity of mucosa from the caecum of germfree rats was 4.5 (Fig 1) The same pH optimum was observed for caecal content from germfree rats, whereas the caecal content from the contaminated rats had a pH optimum of 6.5 (Fig 2)

The content from small intestine of germfree and contaminated rats had a higher β glucuronidase activity at pH 4.5 than at pH 6.5 The same was found in content from the caecum and large intestine of germfree rats, whereas the contaminated rats had a higher activity in these regions at pH 6.5 (Table 1)

The activity of the content from the caecum and the large intestine was roughly the

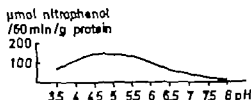


Fig. 1. Activity of β-glucuronidase in caecal mucosa from germfree rats at varying pH.

same in the same rat. The rats monocontaminated with *E. coli* had an activity corresponding to that of the conventional rats. The rats monocontaminated with *S. pyogenes* had an activity approximately 10 per cent of that of conventional rats, whereas the activity of the germfree rats was only 1–2 per cent (Table 1).

The content from the contaminated rats showed a considerable increase in activity from the small intestine to the caecum and a slight decrease for the germfree rats (Table 1).

The mucosa and the rest of the wall had activies which were approximately the same at all levels, but the activity of the mucosal samples tended to be lower than the activity of the rest of the wall (Table 2). The enzyme activities of the contaminated rats were slightly higher than those of the germfree animals (Table 2).

Samples from the mucosa and the rest of the wall were tested at pH 4.5 and pH 6.5. The activity was higher at pH 4.5 for all types of rats.

The number of *S. pyogenes* present in the monocontaminated animals was slightly lower than that of *E. coli* (Table 3). The mean number of bacteria from the small intestine was at least 100 times lower than that of the more distal segments.

DISCUSSION

The observed pH optima of the intestinal β-glucuronidases are in accordance with the values reported by others. The mucosal pH optimum of 4.5 lies in the middle of the reported range from 4.0 to 5.0 (6, 9, 13, 29). Corresponding reports on pH optima of *E. coli*, *S. pyogenes* or intestinal bacterial β-glucuronidase vary from 6.0 to 7.0 (2, 10, 12, 21, 29). The pH optimum of 6.5 observed for caecal content from monocontaminated and conventional rats therefore suggests that the enzyme is of bacterial origin.

When the activities at pH 4.5 and 6.5 are compared, it would appeare that most of the β-glucuronidase in the content of the medium segment of the small intestine from all the rats was of animal origin, whereas the enzyme in the more distal segments from the conventional and monocontaminated rats was mainly of bacterial origin.

The difference in activities at pH 6.5 of the contents from the small intestine and the more distal segments was about 1:50 in the conventional animals. The results may indicate an even greater activity of the bacteral β-glucuronidase in the distal intestinal segments than expected previously (3, 4, 16).

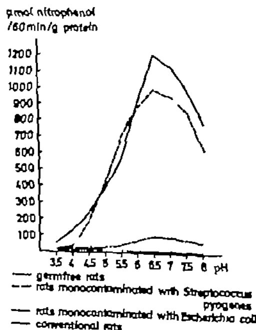


Fig. 2. Activity of β-glucuronidase in caecum content from germfree, monocontaminated, and conventional rats at varying pH.

TABLE 1 *Activity of β-Glucuronidase in Intestinal Content from Germfree Monocontaminated and Conventional Rats at pH 4.5 and 6.5 Activity given as μmol nitrophenol/60 min/g protein*

		Small intestine		Caecum		Large intestine	
		pH 4.5	pH 6.5	pH 4.5	pH 6.5	pH 4.5	pH 6.5
Germfree	mean	28	23	20	16	18	13
	S.D	4.9	5.1	2.4	2.4	3.8	3.6
Streptococcus	mean	38	28	22	100	31	120
pyogenes	S.D	4.5	3.2	5.1	14	6.3	19
	mean	31	21	200	990	270	1100
Escherichia coli	S.D	6.3	6.6	18	59	33	150
Conventional	mean	36	25	240	1100	210	890
	S.D	3.3	2.6	73	200	62	280

TABLE 2 *Activity of β-Glucuronidase in Intestinal Content Mucosa and the Rest of the Intestinal Wall from Germfree Monocontaminated and Conventional Rats at pH Optimum. Activity Given as μmol Nitrophenol/60 min/g Protein*

		Small Intestine			Caecum			Large intestine		
		content	mucosa	wall	content	mucosa	wall	content	mucosa	wall
Germfree	mean	28	140	190	20	140	180	18	150	220
	S.D	4.9	32	33	2.4	38	19	3.8	38	28
Streptococcus	mean	38	200	230	100	210	260	120	210	260
pyogenes	S.D	4.5	35	31	14	38	29	19	28	37
Escherichia coli	mean	31	180	300	990	230	340	1100	310	310
	S.D	6.3	19	59	59	33	61	150	37	43
Conventional	mean	36	180	350	1100	240	350	890	290	360
	S.D	3.3	37	33	200	41	46	280	49	55

(pH optimum is determined to be 6.5 for samples of intestinal content from caecum and the large intestine of the contaminated rats, whereas the pH optimum of the rest of the samples is determined to be 4.5)

TABLE 3 *Number of Microorganisms Present in Content from Different Intestinal Levels of Monocontaminated Rats*

Monocontaminant	Numbers of organisms per gram*		
	Small intestine	Caecum	Large intestine
Escherichia coli	10^{4}–10^{7}	10^{9}–10^{10}	10^{8}–10^{10}
Streptococcus pyogenes	10^{3}–10^{7}	10^{8}–10^{9}	10^{7}–10^{9}

* Each specimen was investigated in triplicate, using tenfold dilution in broth. The numbers indicate absolute range

However the differences may be due to some extent to a difference in the substrate specificities of the animal and bacterial β-glucuronidases (27)

The high activity in the lower intestinal segments of the rats monocontaminated with *E coli* indicates that the level of intestinal bacterial β-glucuronidase activity may be unrelated to a normal flora of anaerobes.

The lower enzyme activity in the rats

monocontaminated with *S pyogenes* may be attributed to a smaller number of bacteria or to a lower enzyme production per organism. The non-inducible nature of streptococcal β-glucuronidase (10) must also be considered.

The β-glucuronidase activity in the stools of newborn babies has been shown to be of the non-bacterial type (26) The present results indicate that intestinal establishment of a β-glucuronidase-producing strain changes the germfree rat from "baby like" to adult.

The activities of the mucosa and the rest of the intestinal wall showed no peak value in the caecum, as reported by *Hänninen et al.* (9) Our results are in accordance with those of *Conchie & MacDonald* (4) who found fairly constant values throughout the alimentary tract.

The tendency for the contaminated rats to have greater activity than the germfree rats in the mucosa and the rest of the wall is probably not due to bacterial β-glucuronidase, as the activity was greater at pH 4.5 than at pH 6.5 It seems possible that the increase may be caused partly by lysosomal β-glucuronidase from leucocytes accumulating in the intestinal wall of the contaminated animals.

The activity of the mucosa of the small intestine observed by us corresponded well with the results of *Koldovsky et al.* (13 14) but was somewhat higher than that observed by *Raychaudhuri & Desai* (19)

The activity of the large intestine was somewhat lower than reported by *Genel et al.* (6) for human rectal mucosa.

The level of β-glucuronidase in the small intestine is of particular importance from a functional point of view (8)

In the present study most of the total intestinal β-glucuronidase content of conventional and monocontaminated rats was found to be located in the lower intestinal segments and was of bacterial origin.

Whether the amount of β-glucuronidase present in the small intestine in the germfree rats is sufficient to split all the glucuronide present is still an open question.

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Acta path. microbiol. scand. Sect. B, 85 277 280 1977

OCCURRENCE OF AN EXTRACELLULAR SERINEPROTEINASE AMONG *STAPHYLOCOCCUS AUREUS* STRAINS

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The production of staphylococcal serineproteinase (Protease I) in 170 *Staphylococcus aureus* strains from human and bovine clinical isolates was investigated. Protease was identified in 57 per cent of the strains by electroimmuno assay

Key words *Staphylococcus aureus* staphylococcal serineproteinase.

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Received 18.I.77 Accepted 6.iv 77

Most staphylococcal strains produce proteolytic enzymes. Baird-Parker reported that 91 per cent of all *Staphylococcus aureus* strains were proteolytic (6) *Martley et al.* (12) suggested a method of classifying staphylococci according to their ability to produce different proteolytic zones on casein agar However they did not isolate any proteolytic enzymes. *Sandvik & Fossum* (14) suggested a classification based on the serological differentiation of staphylococcal proteinases They could show that there are at least *five* different proteinases based on serological identification. However none of these could be isolated and characterized.

Three different proteolytic enzymes from *Staphylococcus aureus* strain V8 have been purified and characterized (3 4 7 9 10) The serineproteinase (Protease I) has an unique specificity cleaving at the carboxy terminal side of glutamic acid and aspartic acid. The thiolproteinase (Protease II) has a specificity resembling that of papain, and the metalloproteinase (Protease III) resembles thermolysin (1) In our laboratory we have been interested mainly in Protease I due to the unique specificity of that proteinase. Peptides derived from proteins hydrolyzed by Protease I are generally large and insoluble (7) and cannot be taken up and metabolized (13) It is thus suggested that Protease I acts together with other proteinases to produce small peptides that can be transported over the membrane. However Protease I could also act as a regulatory enzyme i.e. activation of other enzymes by partial hydrolysis. We were therefore interested in the distribution of this enzyme among *Staphylococcus aureus* strains.

MATERIALS AND METHODS

Strains. A total of 170 staphylococcal strains were examined. 15 of these were international laboratory strains selected for their ability to produce extracellular enzymes and toxins. 134 strains were fresh

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MATERIALS AND METHODS

Strains. A total of 170 staphylococcal strains were examined. 15 of these were international laboratory strains selected for their ability to produce extracellular enzymes and toxins. 134 strains were fresh

human clinical isolates from the National Laboratory of Bacteriology in Sweden, and 21 were isolated from cattle at the National Veterinary Institute Stockholm, Sweden The strains were identified as *Staphylococcus aureus* by microscopical examination and coagulase testing. All strains were phage typed by the standard set of *S aureus* bacteriophages and were tested for DNase production Haemolysin production was tested on horse blood agar plates.

Screening for proteolytic activity was performed on CCY medium (5) supplemented by 1 per cent casein and solidified with 1.5 per cent agar All proteinase positive strains were investigated further by cultivation in a liquid medium in baffled Erlenmeyer shake flasks, as described previously (5) A lactalbumine-yeast extract medium was used (LAY medium) Lactalbumine hydrolysate has been shown to stimulate proteinase production (8) The LAY medium had the same composition as the CCY-medium (5) except that acid casein hydrolysate was replaced by enzymic lactalbumine hydrolysate (Sigma Chemical Company, St. Louis, Mo. U.S.A.) In order to avoid precipitation, this medium must be sterilized at pH 5.5 The pH was adjusted to 7.2 before use. Bacterial growth was determined by dry weight measurements. Samples for proteinase assay were withdrawn at six and eight hours during late logarithmic growth.

Proteinase assay At present no specific substrates for staphylococcal proteinases are available However the three proteinases could be differentiated by an inhibition technique described by *Arvidson* (1) using casein as substrate This technique takes advantage of the fact that Protease III is sensitive to EDTA and that Protease II must be assayed under reduced conditions. Assuming that no proteinases other than Proteinase I II and III exist, then "Protease I activity" means a proteinase insensitive to EDTA and oxidation. In addition to this enzymic test, Protease I was determined by quantitative electroimmuno assay according to *Laurell* (11) as described by *Arvidson* (2) Highly purified Protease I was included in each run as control. The areas of the rockets were measured and the amount of Protease I (µg/ml) was calculated from a standard curve. Protease I antiserum was obtained from rabbits by immunizing them with purified Protease I four times at four weekly intervals.

RESULTS

The production of staphylococcal serineproteinase Protease I of 134 human clinical isolates, 21 bovine clinical isolates and 15 laboratory strains of *Staphylococcus aureus* was examined. Screening for proteolytic activity was performed on casein agar Proteolytic strains usually gave a zone of precipitation around the bacterial streak. This precipitate could either be due to Protease I which is known to give a stable casein precipitate (1) or to some proteinase with broader specificity which at low concentrations could give precipitation (1) For many strains a clearing of the precipitate was observed. This clearing starts near the bacterial streak and proceeds outwards, and is probably due to proteinases with broader specificities (Protease II and

TABLE 1 *Production of Extracellular Proteolytic Enzymes by 170 Strains of* Staphylococcus aureus

	Number of strains producing Protease I	Number of strains producing other proteinases than Protease I	Number of strains with no extracellular proteolytic activity
Total number	97	47	26
DNase positive	94 (97 %)	45 (96 %)	24 (92 %)
Haemolysin positive a)	79 (81 %)	30 (83 %)	4 (15 %)
Phage group			
I	41 (42 %)	18 (38 %)	7 (27 %)
II	11 (11 %)	3 (6 %)	7 (27 %)
III	19 (20 %)	16 (34 %)	7 (27 %)
IV	2 (? %)	0 (0 %)	1 (4 %)
Others b)	16 (16 %)	8 (17 %)	? (8 %)
Non typeable	8 (8 %)	2 (4 %)	2 (8 %)

a) Haemolysis on horse blood agar
b) Including strains belonging to more than one phage type group

III) (1) In the absence of precipitate, clearing of the casein could not be seen. In order to identify strains that only produce proteinases with clearing effects on casein, a 10 per cent solution of perchloric acid (HClO) was poured over the plates. Undigested casein was precipitated by the acid and clearing due to proteolytic activity could be observed. By this technique several additional proteolytic strains were recorded. In total, 85 per cent of the strains were proteolytic and these were cultivated in liquid medium for further studies. All strains grew well and a bacterial dry weight of 7–14 mg per ml was obtained after eight hours of cultivation. The proteolytic activity of the culture supernatants was tested as described in Materials and Methods. Some strains produced very small amounts of proteinase which were only detected after tenfold concentration of the culture supernatants. A proteolytic enzyme similar to Protease I (i.e. insensitive to EDTA and oxydation) was found in 81 per cent of the proteinase positive strains (117 out of 144). However Protease I antiserum reacting material was found only in 67 per cent of the proteinase positive strains (97 out of 144 see Table 1). No correlation was found between Protease I production and the production of DNase and haemolysin. Neither could any correlation be established between Protease I production and phage type. However most proteinase negative strains were also haemolysin negative.

Most human isolates produced small amounts of Protease I (0.5–1 unit per ml) whereas strains isolated from cattle generally were good producers, giving 4–5 units per ml. However no strain was found which gave more Protease I than strain V8, which was originally used for purification and characterization of this enzyme.

DISCUSSION

Extracellular protrolytic activity was found in 144 out of 170 (85 per cent) *S. aureus* strains, which is consistent with the frequency reported by *Baird-Parker* (6). However Protease I was found in only 57 per cent of the strains, thus showing that the proteolytic activity of *S. aureus* should not be regarded as a homogeneous property i.e. the proteolytic activity of different strains may be due to different enzymes. The fact that Protease I was only produced by 57 per cent of the strains also indicates that this enzyme is not essential for growth *in vivo* though it may contribute to the virulence of some strains of *S. aureus*. We have observed that strains from bovine mastitis generally produce very large amounts of Protease I. The ability of Protease I to clot milk may be of some importance in these infections by causing occlusion of the milk glands and glandular ducts. In the case of human isolates, we have so far not been able to correlate the production of Protease I with any particular type of infection.

Support from the *Swedish Medical Research Council* (project number 4513) is gratefully acknowledged. The authors also wish to thank Dr *L. Sjöberg* at The State Bacteriological Laboratory for performing phage typing, and Mrs *U. Björk* and Mrs *M. Tigerholm* for skilful technical assistance.

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Acta path. microbiol. scand. Sect. B, 85 281–285, 1977

MORPHOLOGY AND ULTRASTRUCTURE OF *UREAPLASMA UREALYTICUM* IN AGAR GROWTH

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Black, F T & Vinther O. Morphology and ultrastructure of *Ureaplasma urealyticum* in agar growth. Acta path. microbiol. scand. Sect. B, 85 281–285, 1977

Colonies of *Ureaplasma urealyticum* serotype VI grown on agar were examined by thin sectioning technique. The arrangement of cells within the colonies was less dense and less stratified than that observed in most other mycoplasma colonies. Small, dense and pleomorphic cells were located mainly along the colony borderline towards the agar. Studies of cell morphology after 3 10 and 15 days of growth revealed that such cells were younger than the less dense, mostly ovoid cells seen in the central area of colonies. The ultrastructure of individual cells was similar to that of *U eaplasma* grown in liquid medium, as described earlier featuring geometrical ribosome arrangements and extramembranous coat. Serial sections demonstrated the difficulties involved in discussing cell morphology on the basis of single thin sections. The advantages of the preparation method used for electron microscopy are discussed.

Key words *Ureaplasma urealyticum* morphology ultrastructure, agar growth.

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Received 6.iv.77 Accepted 6.iv.77

Previous studies on the morphology and ultra structure of *Ureaplasma urealyticum* have been performed on organisms grown in liquid medium, using both negative staining and ultrathin sectioning techniques (2, 12, 14 16 17) The findings are essentially identical with those of other mycoplasmas studied (4 5 8)

This paper presents the results of an electronmicroscopic investigation of thin sectioned agar grown colonies of *Ureaplasma urealyticum* fixed *in situ*

MATERIALS AND METHODS

U eaplasma urealyticum serotype VI (3) (Purllo) was cultivated on solid S-medium (1) Plates were incubated at 37° C in moist atmosphere of 95 per cent N_2 + 5 per cent CO for 3 10 and 15 days respectively Colonies were fixed *in situ* in 0.3 per cent glutaraldehyde in veronal-acetate buffer containing 0 01 M $CaCl_2$, pH 6.1 (VA buffer) for one hour at room temperature. After removal of excess glutaraldehyde and drying of the plates for 30 minutes at 37 C, colonies selected for electron microscopy were covered with a thin layer of melted (45 C) 1 per cent Noble agar (Difco) in VA buffer After solidification, small agar blocks with single colony embedded were cut and fixed further first in 3 per cent glutaraldehyde in VA buffer for one hour at room temperature, then in a 1 per cent

OsO_4 solution containing yeast extract—sodium acetate—peptone medium (6) overnight. Afterwards blocks were immersed in 2 per cent uranyl acetate for one hour (13) and subsequently dehydrated in a graded series of acetone-H_2O mixtures. Blocks were embedded in Vestopal W being oriented in such a way that thin vertical cross sections through the colonies were obtained using an LKB Ultrome – III microtome. Sections were examined after post staining with magnesium uranyl acetate and lead citrate in a JEOL JEM 100 B electron microscope.

RESULTS

As expected from the "fried egg" appearance of the *Ureaplasma urealyticum* colonies in the light microscope cross sections (Figs 1 & 2) reveal a narrow peripheral and a central approximately hemispherical part of the colonies. The surface growth can be distinguished from agar growth as a narrow zone of less dense cell population in the uppermost part of the colonies. In contrast to most other mycoplasmas studied further distinct zones of different population density and cell morphology cannot be observed within the colonies. However small dense often elongated cells are located mostly along the borderline of the agar grown part of the colonies, whereas the larger and less dense cells are found mainly in the central area of the hemispherical part.

Figs. 3, 5 and 6 show the peripheral part of the surface growth of colonies incubated for 3, 10 and 15 days, respectively. The cells of the young colony (Fig 3) are pleomorphic and frequently elongated or filamentous, often with club-shaped terminal parts. Fig 4 shows a high magnification of a filamentous part of a cell. It consists of an electron dense layer interposed between two transparent triple-layered cell membranes, both of which possess a hairlike outer surface layer. The spacing between the two transparent membrane layers is approximately 7 nm and the thickness of the surface layer is of the same magnitude. From Figs. 5 and 6 it can be seen that on ageing the peripheral cells become more uniformly ovoid and less dense and empty cells are observed with increasing frequency.

Although not shown on the micrographs, corn-cob-like arrangements of ribosomes were often observed, but only in young colonies.

The cell in Fig 7 reveals clearly an extramembranous layer showing a well-defined spike like substructure that seems to be rooted in the outer layer of the membrane and to

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Fig 1 Cross section of 3-day-old agar grown colony of *U urealyticum* serotype VI. In the uppermost part of the colony can be seen a relatively narrow zone of less dense cell population indicating surface growth. × 677. Bar represents 25 µm.

Fig 2 Higher magnification of the bottom part of the colony in Fig 1. Dense elongated cells can be seen in the lower part of the segment, in contrast to the larger ovoid and less dense cells which appear nearer the centre of the colony. × 4650. Bar represents 5 µm.

Fig 3 Peripheral part of 3-day-old colony (surface growth). Dense elongated cells, often with club-shaped terminations, can be seen together with very long apparently filamentous cells. × 4500. Bar represents 5 µm.

Fig 4 High magnification of a segment of a filamentous organism, showing a narrow electron dense layer interposed between two triple-layered membranes, both possessing an extramembranous surface layer. × 144 000. Bar represents 100 nm.

Fig 5 Peripheral part of 10-day-old colony. Most cells are ovoid and rather uniformly dense. A small number of cells containing loose cytoplasm can also be seen. × 18 000. Bar represents 1 µm.

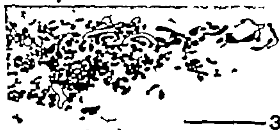
Fig 6 Peripheral part of 15-day-old colony. The frequency of organisms with loose cytoplasma has increased. Some almost empty cells can be observed. × 18 000. Bar represents 1 µm.

Fig 7 High magnification of a single *U urealyticum* cell. The extramembranous layer is seen to radiate out from the outer layer of the triple-membrane being apparently rooted in this layer. The interior of the cell is filled with nearly uniformly distributed ribosomes. × 168 000. Bar represents 100 nm.

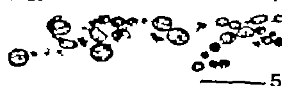
Fig 8 a–f Serial sections through peripheral part of 3-day-old colony. Section thickness was estimated to be about 60 nm for each section. Between sections a and b, two sections of the same thickness (not shown) were interposed, and between section d and e one section was interposed. The series shows the very variable appearance of a cell (arrow) viewed in sections at different levels. × 15 600. Bar represents 1 µm.


1


2

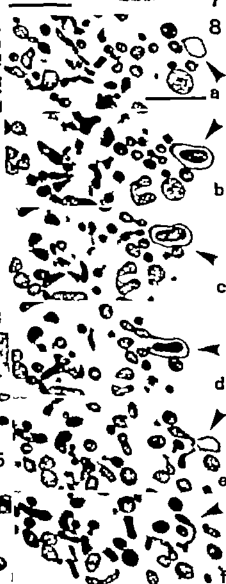

3


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6


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8
a
b
c
d
e
f

form an integral part of it. The interior of the cell contains rather uniformly distributed *ribosomes*.

Figs 8 a–f show serial sections through the peripheral part of a 3-day-old colony. Noteworthy is the appearance of the same cell in the different sections. What seems to be a circular cell, a circular cell containing a round cell in its interior, a highly pleomorphic cell, and a very filamentous cell with swellings in the terminal parts, are actually all the same cell at different section levels.

DISCUSSION

The method of specimen preparation used in this study, in particular *in situ* fixation of agar embedded colonies, seems to be suitable for ultrastructural studies of *Ureaplasma urealyticum* since individual cells appear to be structurally well preserved. The three layers of the cell membrane are easily recognized (Fig 7) and the extramembranous layer is uniform in thickness (Figs. 4 & 7) indicating the absence of large amounts of adsorbed medium components. Cells prepared this way are not exposed to the stress of centrifugation. Furthermore, the method is less laborious and requires less medium than is necessary when mycoplasma grown in liquid medium are to be processed for electron microscopy.

The gross colony morphology of *U. urealyticum* (Fig 1) does not differ essentially from that of other mycoplasmas examined (7, 9, 10). The surface layer is the only clearly outstanding layer of cells, the cell population density being quite uniform throughout the agar embedded part of the colonies. In colonies of *M. orale*, *M. pneumoniae* and *M. salivarium* several distinct zones of varying population density and cell morphology have been described (7, 9).

It is reasonable to assume that the relatively small, dense and highly pleomorphic organisms observed along the borderline of *U. urealyticum* colonies (Fig 2) are younger than the less dense, mostly ovoid organisms seen in the central area of colonies. This assumption is supported by the observation of cells in the peripheral part of the surface growth of colonies grown for different lengths of time (Figs. 3, 5 & 6). The peripheral cells in the young colonies are mainly small, dense and often elongated, but become less dense and more rounded on prolonged incubation.

The filamentous-looking part of elongated cells often showed a uniform width of about 25 nm over a distance of more than 1 µm (Figs. 3 & 4). This makes it highly improbable that these structures are true filaments of a more or less cylindrical outline. They are more probably band-shaped or collapsed parts of mycoplasma cells, as is also suggested by the serial sections (Fig 8). Arrangements of double unit membranes, resembling those observed in the present study, have also been observed in *M. salivarium* colonies (7).

Whitescarver & Furness (16) have ascribed the appearance of filamentous forms of *U. urealyticum* to inadequate fixation of the organisms. Since, as demonstrated in the present study, the filamentous-looking forms exist only in young colonies, the fixation procedure used througout this investigation probably cannot account for these forms. Filamentous forms of *U. urealyticum* cells from broth cultures have been observed previously both by thin-sectioning and negative staining techniques (2, 14).

The extramembranous layer surrounding the cells seems to be composed of spike like subunits (Fig 7), as has been reported also for liquid grown organisms (2, 16). The spikes appear to form an integral part of the outer layer of the cell membrane (Fig 7). This extramembranous layer has been shown recently to contain glucosyl-like residues (11).

Corn-cob-like ribosome arrangements (2, 15, 16) were observed frequently in cells of 3-day-old colonies but never in older colonies. Possibly such arrangements preferentially show up in cells with a high rate of protein synthesis.

Like *Black et al.* (2) but unlike *Whitescarver & Furness* (16) we did not observe any vacuoles in either young or older cells.

The present study has not established with certainty the mode of replication of *U. urea-*

lyticum. *Whitescarver & Furness* have suggested that replication occurs by production of one or two buds which are eventually separated from the mother cell (16) However the serial sections (Fig 8) demonstrate that extreme care should be exercised in assigning a definite morphology to mycoplasma cells or in drawing conclusions regarding their replication on the basis of the appearance of cells in single thin sections.

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Acta path. microbiol. scand. Sect. B 85 286–288 1977

BRIEF REPORT

PIGMENT PRODUCTION IN GROUP B STREPTOCOCCI

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Holth Haug R. & Soderlund E. Pigment production in group B streptococci. Acta path. microbiol. scand. Sect. B 85 286–288 1977

Most haemolytic group B streptococci produced a yellow to orange pigment in Colombia agar and in some Todd Hewitt broths The composition of the broth medium was important. Trypsin inhibited pigment production, but had no effect on pigment already produced. Optical density measurement revealed three main peaks at 420 260 and 215 nm respectively with pigment producing strains. Nonpigmented strains gave only the two peaks at 260 and 215 nm.

Key words Streptococcus group B pigment production

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Received 7.iii 77 Accepted 16.iv 77

Pigment production specific for group B streptococci has been reported by several workers (1 3 4 5) and the pigment may be useful for the identification of group B strains. For this purpose, stab culture in Columbia agar has been recommended (1 4 5) We have observed group B strains which characteristically elaborate yellow to orange pigment in different media, and in this study attention is drawn to certain factors which may influence the pigmentation capacity

Material and Methods

The pigment production of 56 human group B strains collected from different sources and 15 bovine strain from mastitis in cows was studied in Columbia agar The medium was prepared as proposed by *Fallon* (1) The streptococcus strains had been grouped by conventional methods (2 6)

For comparison 44 group A, 17 group C 14 group G and 28 group D strains, all isolated from routine clinical specimens, were also examined. The organisms were stabbed into the agar and cultivated aerobically for 18 hours at 37 C The pigment production was read immediately after incubation

The pigmentation capacity of reference strains of group B serotypes Ia (090) Ib (H36B) Ic (A909) II (18RS21) III (D136C) and one bovine strain Ic was studied in four different broths.

1) Todd Hewitt broth (THH) made from horse meat (20 g Proteose Peptone, Difco, 2 g glucose 1 g Na_2HPO 12 H_2O 2 g $NaHCO_3$ and 2 g NaCl per l pH 8 0)

2) Todd Hewitt broth (THB) made from beef (20 g Bacto Peptone Difco 2 g glucose 1 g Na_2HPO_4 12 H_2O 2 g $NaHCO_3$ and 2 g NaCl per l pH 7 0)

3) Oxoid Todd Hewitt broth (THO)* with 1 per cent Neopeptone

4) Brain Heart Infusion broth (BH) Difco.

Broth cultures of the strains were incubated for 18 hours aerobically with and without CO_2 and anaerobically in a mixture of H_2 and CO_2 (Gas Pak system) Addition of 0.6 per cent trypsin was tried in order to examine the effect on pigment production. The sediments from broth cultures were washed in physiological saline and treated with 0 2 M HCl and 0 1 M NaOH. The pigment extracted in this manner was water soluble the colour varied from yellow to orange and was almost red in the bovine Ic strain. The pigment

* The composition kindly provided by Dr H R *Marted* Cross Infection Reference Laboratory Colindale, London.

TABLE 1 *Pigment Production by Group B Strains in Colombia Agar*

	Human strains	Bovine strains
Pigment	52 betahaemolytic 1 nonhaemolytic	5 betahaemolytic
No pigment	2 betahaemolytic 1 nonhaemolytic	2 betahaemolytic 8 nonhaemolytic
Total	56	15

TABLE 2. *Pigment Production by Group B Strains in Four Different Broths*

Strains	THH	THO	THB	BH
I (090)	+ +	+ (+)	—	—
Ib (H36B)	(+)	(+)	—	—
Ic (A909)	+ +	+ +	—	—
II (18R521)	+ (+)	+ (+)	—	—
III (D136C)	—	+	—	—
Ic, bovine strain	+ + + +	+ + + +	+ +	+ (+)

(+) weak reaction.

precipitated on addition of HCl and was redissolved on neutralization. The optical density was measured in Beckman spectrophotometer (DB-G) at different wavelengths from 190 to 600 nm.

Results and Discussion

None of the group A, C, D and G strains examined produced pigment in Columbia agar. Table 1 shows the results for the group B strains. Under certain conditions group B strains appear to loose the properties of haemolysing blood and producing pigment (3). Of all our strains only one nonhaemolytic group B strain yielded pigment in Colombia agar. Table 2 shows the pigment production in four different broths.

Organisms from the serotypes Ia, Ib, Ic and II produced pigment only in THH and THO, serotype III only in THO. The bovine Ic strain, on the other hand, yielded pigment in all broths tested. There was no significant difference in pigment production between aerobically and anaerobically cultivated organisms. The addition of 0.6 per cent trypsin to the broth cultures before incubation inhibited the production of pigment.

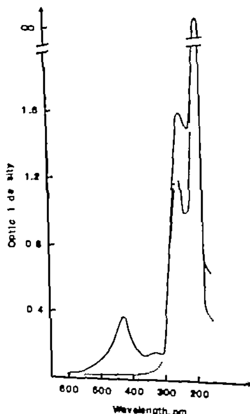


Fig. 1. Optical densities, pigmented and nonpigmented strains serotype I. —— Pigmented strain, - - - nonpigmented strain.

A similar inhibition was observed in broths pre-incubated for 2 hours at 37 C with the same amount of trypsin and then inoculated after inactivation of trypsin. The trypsin was inactivated by steaming the broths for 15 min. These observations seem to indicate that the presence of trypsin inhibits the pigment production by destroying substances in the medium which are necessary for this reaction. Addition of trypsin had no effect, however on pigment already produced. The optical densities of the pigment solutions are shown in Fig. 1 There were three main peaks, one at 420 nm and two in the U V region, at 260 and 215 nm respectively The peak at 420 nm varied according to colour intensity as observed in the cultures. Supernatants from nonpigmented colonies gave only the two peaks at 260 and 215 nm respectively

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Some recently published supplements to

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Acta path. microbiol. scand. Sect. B 85 289–295 1977

INDUCTION OF LATENT HERPES SIMPLEX VIRUS TYPE 2 INFECTION IN HUMAN CERVICAL EPITHELIAL CELLS *IN VITRO*

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Vesterinen, E., Leinikki, P. & Saksela, E. Induction of latent herpes simplex virus type 2 infection in human cervical epithelial cells in vitro. Acta path. microbiol. scand. Sect. B, 85: 289–295, 1977.

An in vitro method was used to induce non-productive herpes simplex virus type 2 (HSV 2) infections in human ecto- and endocervical epithelial cells. Adenine arabinoside (ara-A) could prevent the replication of HSV 2 in ecto- and endocervical cells and after removal of ara-A from growth medium rapid destruction of explants with development of infectious virus was observed. When ara-A was not withdrawn until 4 days or later after in vitro infection the morphological, immunofluorescent and virological studies detected no infection. However 2–3 weeks after inoculation there were in some cultures morphological as well as immunofluorescent signs of HSV infection without demonstrable infectious virus, indicating an incidental abortive nature of the infective process.

Key words: Herpes simplex virus type 2; latent infection; cervical epithelial cells; in vitro.

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Received 7.ix.76 Accepted 29.iii.77

Herpes simplex virus type 2 (HSV 2) seems to be a quite rare inhabitant of the human uterine cervix (16) although 10–20 per cent of patients in different populations possess antibodies against it (4). This virus, like also other members of the herpes group of viruses induces latent infections. The clinical manifestations of the latent virus are observed as periodical eruptions of genital vesicles and ulcerations. The latent HSV 2 has recently been shown to reside in the local sensory ganglia of the sacral region (3). However if HSV 2 is an etiological factor in cervical carcinoma as suspected on the basis of in vitro studies (10) and clinico-epidemiological investigations (7), a non-lytic relationship between the virus and cervical epithelial cells is necessary.

In in vitro experiments the lytic character of an HSV infection can be diminished by introducing specific anti-HSV antibodies to culture fluids, by depletion of certain nutrients from the growth medium or by using low or high temperature incubation. These methods, however, usually permit the growth of small amounts of infective virus as a sign of productive infection.

Using the cytosine arabinoside (ara-C) the productive cycle of HSV 2 infection

could be prevented in fibroblasts, as first demonstrated by *O'Neill et al* (8). Also another nucleoside analogue, adenine arabinoside (ara A) is able to inhibit the infective cycle of HSV 2 (9).

We have used ara A to induce latent HSV 2 infections in cultured human ecto-and endocervical epithelial cells. In order to verify the nonproductive character of the infection morphological, immunofluorescent and virological methods were utilized.

MATERIALS AND METHODS

Ecto- and endocervical epithelial cell cultures were initiated from patients which were hysterectomized for benign gynaecological diseases at the I II Departments of Obstetrics and Gynaecology, Helsinki University Central Hospital. Before hysterectomy the cervicovaginal Papanicolaou smear was normal and this was confirmed after operation by histologic examination of cervical tissues. The methods used for the preparation of cultures and culture of epithelial explants have been described earlier (13 15). The cells were grown in Ham's F 10 medium (Flow Laboratories, Irvine Scotland) supplemented with 10 per cent foetal calf serum (Flow) antibiotics and glutamine as described in detail earlier (15). Explants were cultured on coverslips in 50 mm petri dishes in humidified air atmosphere with 5 per cent CO_2 at 37 C.

Virological Techniques

Infection of explants in vitro with HSV 2. HSV 2 (Curtis E 304) originally supplied by Dr H H *Dowdle* Atlanta Ga was used. The propagation of virus for experiments is described in detail elsewhere (14). A cell free virus suspension released by freezing thawing and crude centrifugation was used as an inoculum. The amount of inoculum varied between 1 × 10 and 1 × 10 TCID coverslip in 0.2 ml, however 1 × 10 $TCID_{50}$ was mostly used. The adsorption time was 1 h after which each culture was washed three times with phosphate buffered saline (PBS) 2 ml of growth medium was added, and the cultures were maintained at 37 C in a 5 per cent CO humid environment.

Virus assay. The assays of infective virus from growth medium of explants were performed on continuous monkey kidney cells (BSC-1) by plaque assay under carboxymethylcellulose (CMC) overlay as described earlier (5). In cases where HSV 2 was to be assayed from growth medium containing adenine arabinoside the BSC-1 cell were carefully washed with PBS after incubation.

Adenine Arabinoside (ara A)

Vidarabine 5'phosphate ester disodium, a salt of ara A was used. The agent was supplied by Dr *J A L Gorringe* of Parke Davis & Co Pontypool Wales. For blocking experiments ara-A was diluted in growth medium to give appropriate concentrations. Concentrations varied between 5 µg/ml and 30 µg/ml however 20 µg/ml was mostly used.

Fluorescent Antibody Technique (FA)

Coverslips with infected explants were washed with PBS and fixed after air-drying with acetone at —20 C for 15 min. Staining was performed as described in detail elsewhere (5). Rabbit immune sera were originally produced by ocular inoculation with HSV 2 strain Curtis E 304 grown in rabbit kidney cells. Serum in a dilution of $^1/_{16}$ in PBS was inoculated on the explants for 30 min at 37° C. After washing with PBS and distilled water the explants were inoculated with commercially available fluorescein isothiocyanate (FITC) conjugated antirabbit immunoglobulin (Wellcome & Co London) in the working dilution of $^1/_4$ in PBS and incubated as above. Amido black, 1 mg/ml, was added as counterstain. For controls uninfected explants from each experiment were treated with virus antiserum and conjugate as above. For fluorescence control, explants from each experiment were treated with acute phase serum and handled as above. A Leitz Ortholux microscope with dark field condenser objective 10 × with a HBO 200 mercury lamp and Balzer's FITC interference filters was used.

Cytological Techniques

The coverslips were washed with PBS, fixed with propanol fixative (Pro-FiXX, Scandialab A/S Copenhagen, Danmark) and stained according to the original Papanicolaou staining method.

RESULTS

In preliminary experiments an adequate blocking effect without morphologically noticeable disturbance in cell proliferation could be regularly obtained with an ara A concentration of 20 µg/ml in growth medium. An example is given in Table 1 of the development of cytopathic effect (CPE) (Fig. 1) at different concentrations of ara A. A concentration of 20 µg/ml was capable of blocking productive infection if the virus input dose did not exceed 1 × 10 $TCID_{50}$ (Table 2). The activation of productive infection after withdrawal of ara A was greatly dependent

TABLE 1 *Development of HSV 2 Induced) Cyt pathic Effect (CPE)[b]) in Ectocervical Epithelial Cell Cultures) at Different ara-A Concent ations*

Post-infection time (h)	5 μg/ml CPE	10 μg/ml CPE	20 μg/ml CPE	30 μg/ml CPE	Control CPE
0	—	—	—	—	—
20	+	+	—	—	+
30	+	+	—	—	+ +
46	+ + +	+ +	—	—	+ + +
72	+ + +	+ +	+	+	+ + +
96	+ + +	+ +	+	+	+ + +

) Virus inoculum 10^3 $TCID_{50}$/coverslip. Concentration of ara-A in growth medium is stated in each column.

b) CPE + = not more than ¼ of the area of explants show CPE.
CPE + + = ¼–½ of the area of explants show CPE.
CPE + + + = over half of the area of explants show CPE.

) A erage area of explants = 3–4 low power fields (2.5 ×) per culture



Fig 1 Multinucleated giant cells with "ground glass" nuclear appearance (Papanicolaou stain × 550)

of the treatment time In three ecto- and endocerv ical cultures observed the activation of infection occurred when the ara-A treat ment time was 72 *h* or less. An example is presented in Table 3 for ectocervical cells and in Table 4 for endocervical cells. In cases where the blocking treatment was continued for longer periods no morpho ogical signs of HSV 2 infection were present and infectious irus could not be recovered (Table 5) with the exception of two ectocervical cultures in which morphological signs of herpes simplex infection were *detectable* In one culture out of the five followed cell fusion was observed 14 days after in vitro infection (Fig. 2) and in another 21 days after infection, but in fectious virus was *not recovered* from the growth medium. In none of the six endocervical cultures observed could morpholog cal changes compatible with HSV infection be seen.

Cytological Features and FA Pattern of HSV 2 Infected ara A Treated cells

In ara-A treated ecto- and endocervical explants the HSV 2 induced CPE, when present, resembled that observed in untreated *cultures*. There was focal fusion of cells, fragmentation of chromatin and "ground glass" nuclear formation, rarely followed by intranuclear inclusions (Fig 1) *FA* regularly detected *virus* specific proteins in some cells in explants infected with HSV 2 and blocked with ara A but in which productive infection was not observed. Cytoplasmic fluorescence predominated in these (*Fig* 3) whereas in cultures in which the blocking effect had failed there was strong fluorescence throughout the cells (Fig 4) Uninfected cultures treated with ara A failed to show similar fluorescence.

TABLE 2 *Effect of Different Input Doses of HSV [a] on ara-A [a]) Treated Ectocervical Explants[b])*

Post infection time (h)	ara-A treated cells				Control cells	
	10^3		10^3		10^3	
	CPE [c])	PFU/ml growth medium	CPE [c])	PFU/ml growth medium	CPE [c])	PFU/ml growth medium
0	—	0	—	0	—	0
20	+	0	+	0.02×10^3	+	$\sim 1.0 \times 10^3$
28	+	0	+ +	0.10×10^3	+ +	$\sim 5.0 \times 10^3$
48	+	0	+ + +	$\sim 5.0 \times 10^3$	+ + +	$> 5.0 \times 10^3$

a) 20 µg/ml of growth medium.

b) Average area of explants = 3–4 low power fields (2.5 ×) per culture Input dose is stated as $TCID_{50}$ titre in each column heading.

c) See footnote b in Table 1

TABLE 3 *Blocking of HSV-2[a]) Infection by ara-A[b]) in a Human Ectocervical Epithelial Cell Culture and Appearance of Infectious Virus after withdrawal of ara-A (72 h after Infection in vitro)*

Post-infection time (h)	ara A treated cultures[c])		Control cultures[d])	
	CPE [e])	PFU/ml growth medium covering explants	CPE	PFU/ml growth medium covering explants
0	—	0	—	0
20	—	0	+	$> 1.5 \times 10^3$
30	—	0	+ +	$\sim 5.0 \times 10^3$
46	+	0	+ + +	$> 5.0 \times 10^3$
72	+	0	D[f])	ND[g])
96	+	0.16×10^3		
120	+ +	0.73×10^3		
144	+ + +	$> 1.5 \times 10^3$		
168	+ + +	$\sim 5.0 \times 10^3$		

a) Input dose 1×10^3 $TCID_{50}$.

b) 20 µg/ml of growth medium. Withdrawal of ara A after 72 h is indicated by the broken line.

c) Average area of explants = 3 low power fields (2.5 ×)

d) Average area of explants = 4 low power fields (2.5 ×)

e) See footnote 2 in Table 1

f) D = explants destroyed by HSV 2.

g) ND = not done.

DISCUSSION

The results of the present study show that human ecto- and endocervical epithelial cells are suitable targets for investigations of HSV 2 latency in epithelial cell system Our previous studies (14 15) have shown that human cervical epithelial cells can support the replication of herpes simplex virus type 2 (HSV 2) and cytomegalovirus (CMV) It has been demonstrated that sacral sensory ganglia can harbour latent HSV 2 (3) Spontaneous reactivation of HSV 2 does not occur

TABLE 4 *Blocking of HSV-2a) Inf ction by ara-Ab) in Human Endocervical Epithelial Cell Culture and Appeara of Infectious Virus after withdrawal of ara-A (72 h after Infection in vitro)*

Post-infection time (h)	ara-A treated culturec)		Control culturec)	
	CPE	PFU/ml growth medium covering explants	CPEd)	PFU/ml growth medium covering explants
0	—	0	—	0
20	+	0	+	0.02 × 10³
48	+	0	+ +	~ 2.5 × 10³
72	+	0	+ + +	> 2.5 × 10³
96	+	0.05 × 10³	De)	0.10 × 10³
120	+	0.12 × 10³		
144	+ +	0.50 × 10³		
172	+ + +	~ 1.25 × 10³		

) Input dose 1 × 10³ $TCID_{50}$

b) 20 μg/ml of growth medium. Withdrawal of ara-A after 72 h is indicated by the broken line.

) A erage area of explants = 2.5 low power fields (2.5 ×)

l) See footnote 2 in Table 1

) D = explants destroyed by HSV-2.

TABLE 5 *Example of Long Term ara-A) T atment on Human Ecto-b) and Endocervicalc) Epithelial Cell Culture I fected with HSV 2d) N Infectious Virus Detectable after Withdrawal of ara-A (150 h after Inf ction in vitro)*

Post-infection time (h)	ara-A treated ectocervical culture		Control culture		ara-A treated endocervical culture		Control culture	
	CPEe)	PFU/ml growth medium	CPE	PFU/ml growth medium	CPE	PFU/ml growth medium	CPE	PFU/ml growth medium
0	—	0	—	0	—	0	—	0
24	—	0	+	0.50 × 10³	—	0	—	0
44	+	0	+ + +	~ 5.0 × 10³	—	0	+	0.08 × 10³
60	+	0	+ + +	> 5.0 × 10³	+	0	+ +	~ 1.25 × 10³
72	+	0	Df)	NDg)	+	0	+ +	0.86 × 10³
90	—	0			—	0	+ + +	0.43 × 10³
150	—	0			—	0	+ + +	0.36 × 10³
7 days	—	0			—	0	D	ND
21 days	—	0			—	0		

) 20 μg/ml of growth medium. Withdraw l of ara-A after 150 h is indicated by the broken line.

b) Average area of explants = 4 low power fields (3 ×) per culture.

c) Average area of explants = 3 low power fields (2.5 ×) per culture.

d) Input dose 1 × 10³ $TCID_{50}$

) See footnote 2 in Table 1

f) D = explants destroyed by HSV 2.

g) ND = not done



Fig 2 Cell fusion in a HSV 2 infected and ara-A treated ectocervical explant, 14 days post infection (Panamicolaou stain × 520)



Fig 3 FA positi e cell in an ectocervical explant in which HSV 2 infection has been blocked with ara A (× 75)



Fig 4 Ectocervical cells presenting HSV-specific immunofluorescence in an explant nfected with HSV 2 and treated with ara A (× 150)

from cervical epithelial cells obtained from HSV 2 seropositive patients and maintained in culture environment (14) However at least in one case (1) activation of a HSV strain has occurred in a cell culture obtained from cervical carcinoma.

In order to induce non-productive HSV-2 infections in cervical ep thelial cells, adenine arabinoside (ara A) was used This antiviral agent (9-β D arabinofuranosyladenine) can prevent the replication of DNA containing viruses, but permits appearance of virus specific antigens on the surface of cells (11)

We treated human ecto- and endocervical epithelial cells with ara A after incubation with HSV 2 for 1 h. In concentrations used in these experiments ara A did not prevent the growth of cervical epithelial cells. The possibility to induce non productive infections was dependent besides ara A concentration also on HSV 2 input dose. Most suitable conditions for non productive infection were regularly obtained when input dose 1 × 10 $TCID_{50}$ and ara A concentration of 20 μg/ml were used. In cultures treated as indicated above, however some morphologically altered cells as well as immunofluorescent studies indicated that virus specific alterations occurred although infectious virus did not appear Spontaneous reactivation of HSV 2 regularly occurred if the antiviral agent was removed from the growth medium before 72 h s treatment After longer treatment times with ara A, productive infection was not seen to follow the removal of the blocking agent although some cultures were under observation for several weeks In some cells, however immunofluorescence with formation of syncytium foci without demonstrable production of infectious virus seemed to indicate an abortive viral cycle The possibility however remains that the amount of infectious virus was too small to be detectable from the growth medium by methods used in the present work

The etiologic role of HSV 2 in cervical cancer is still unresolved. Several recent studies, however suggest that HSV 2 may be one of the causative agents responsible for malig

nancy (1 2 6, 12) The possibilty to induce non-productive HSV 2 infections in cervical epithelial cells provide one way where long term relationship between the virus and the relevant target cells system can be investigated.

Th study w supported by grants from *Finnish Cancer Society The Finnish Cult a. Foundation and the Paulo Foundation*

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Acta path. microbiol. scand. Sect. B 85 296–302 1977

RAPID DIAGNOSIS OF INFLUENZA A INFECTION BY IMMUNOFLUORESCENCE

Methodological problems and clinical material

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Olding-Stenkvist, E. & Grandien, M. Rapid diagnosis of influenza A infection by immunofluorescence. Methodological problems and clinical material. Acta path. microbiol. scand. Sect. B 85 296–302 1977

Indirect immunofluorescence (IF) on cell spreads from nasopharyngeal secretions (NPS) was used for the rapid diagnosis of influenza A infection and was compared with IF on monkey kidney cells infected by NPS. The clinical diagnosis of influenza A infection was confirmed by serology in 32 of 40 patients. In 27 of the 32 patients (84 per cent) the diagnosis was achieved by IF on cell spreads of NPS. In 13 of 15 (87 per cent) subjects with positive serology the early appearance of influenza A virus antigen was revealed by indirect IF on infected monolayer cells. No false positive specimens were found among serologically negative subjects by either method. Consequently the reliability of IF on cell spreads of NPS is very similar to IF on infected cell cultures, but offers a much quicker diagnosis (3–4 h as compared to 1–3 d). Monovalent anti human IgG FITC should be used instead of polyvalent anti-human Ig FITC, as the latter contains anti IgA which may adhere to intracellular IgA in the epithelial cells of NPS and thereby cloak the viral antigen.

Key words: Influenza A infection; rapid diagnosis; immunofluorescence.

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Received 5.iii.76 Accepted 4.iv.77

The immunofluorescence (IF) technique applied to cell specimens from the upper respiratory tract has been used for the rapid diagnosis of influenza (reviewed in ref. 3). The results have varied. Good agreement (80–99 per cent) between the results of IF and virus isolation in tissue culture and/or serology has been reported by some authors (2, 4, 5, 6, 8, 9). However, the occurrence of a high percentage (14–39 per cent) of positive IF without positive serology reported by others (11, 12, 13) has raised doubts regarding the reliability of the method.

The present study was undertaken with the aim of evaluating the reliability of the rapid diagnosis of influenza A by IF. Firstly, the IF technique was used to identify influenza virus antigen in cell spreads from nasopharyngeal secretions. Secondly, influenza virus antigen was identified in tissue culture after a short term incubation following inoculation with nasopharyngeal secretions.

MATERIAL AND METHODS

Patients

The study was performed during an outbreak of influenza A in 1975 and included 40 adult patients, 19 males and 21 females, admitted to the Department of Infectious Diseases in Uppsala in the acute stage of an influenza-like illness, with fever, respiratory symptoms, headache and generalized aching of muscles

Nasopharyngeal Secretions (NPS)

Nasopharyngeal secretions were obtained by aspiration from the nasopharynx of 40 patients.

Cell deposits of NPS on microscope slides for IF The NPS from each of 40 patients was dispersed in 5 ml phosphate-buffered saline (PBS) washed and centrifuged twice. The pellet was resuspended in 1.5 ml PBS and the cells were applied to small areas of 5–6 microscope slides by cytocentrifuge, fixed in anhydrous acetone for 2 minutes at 20 C and stored at —20° C if not used the same day

NPS from five individuals with upper respiratory infection of unknown aetiology and from four healthy individuals served as controls.

Short-term cultivation on cover slip cultures for IF NPS from 18 of 40 patients was dispersed in 2 ml Eagle minimum essential medium (MEM) containing antibiotics, and 0.1 ml was inoculated in triplicate on cover slip-cultures of monkey kidney cells in Leighton tubes. The cultures were harvested after 18 42 and 66 h and fixed in acetone (see above)

Conventional virus isolation in tissue culture tube 0.1 ml of NPS suspended in MEM containing antibiotics was inoculated into each of 4–6 tissue-culture tubes with primary monkey kidney cells and tested for haemagglutination (HA) with chicken erythrocytes when the cytopathic effect (CPE) became visible If the HA test was positive, the virus was identified by the haemagglutination-inhibition test (HI) using subtype or strain-specific standard sera. Even if CPE did not emerge, the cultures were tested for HA on day 10 of culture.

Sera

Blood samples for serological tests were collected during the acute and convalescent phases from all 40 patients. Sera were assayed for complement fixing (CF) antibodies according to *Sever* (10). A fourfold or greater rise of antibody titre was required for diagnosis

Immune Reagents

Human anti-influenza A2 sera Acute and convalescent sera from a human case of influenza A2 infection were used as negative control and positive serum, respectively The reciprocal titres of CF antibodies were 5 and 160 There were no detectable CF antibodies to influenza B, adenovirus, or parainfluenza virus types 1 2 and 3 and only a weak reaction to RS virus antigen in the 1.5 dilution. The sera were diluted 1 20 before use.

Sheep anti-human IgG FITC conjugate A sheep anti-human IgG immunoglobulin labelled with FITC (Wellcome lot K 7642) was used. The protein content was 7.3 mg per ml and the antibody protein 1.59 mg per ml. The F/P molar ratio was 3.0 The working dilution was 1.5

Sheep anti-human Ig FITC conjugate The Ig fraction of a sheep anti-human Ig serum conjugated with FITC was obtained from the Department of Immunology National Bacteriological Laboratory Stockholm It contained 7.1 mg protein per ml, corresponding to 2.6 mg of anti-IgG protein per ml. As stated by the manufacturers, it is a polyspecific antiglobulin which reacts with the major classes of Ig (IgG IgM, IgA) The F/P molar ratio was 1.9 It was diluted 1 10 before use.

Sheep anti-human IgA FITC conjugate The Ig fraction from hyperimmune sheep sera against human IgA, labelled with FITC (Department of Immunology National Bacteriological Laboratory Stockholm) was used. The protein content of the conjugate was 5.4 mg per ml and the F/P ratio 5.7 It was used in the tests diluted 1:5

Immunofluorescence Staining Procedure and Microscopy

Direct and indirect IF were performed as described previously (7 9) A Leitz Orthoplan microscope, equipped with incident illumination according to Ploem, was used. Excitation filters were BG 12 and interference filter KP 490 and BG 38 was used as red absorbing filter. K 510 was used as barrier filter

Control of Reagent Sera and Conjugates of IF in Model Assays

Cover slip-cultures of monkey kidney cells in Leighton tubes were inoculated with 0.1 ml of suspension containing 100 $TCID_{50}$ of influenza A2 virus. After 18 hours incubation at 37 C the cover slips were fixed in anhydrous acetone. The cover slips were stained in chessboard fashion with sera and conjugates in twofold dilutions starting at 1.5. Cover slips incubated with PBS instead of sera were also included in the test. The highest dilutions of the human anti-influenza A2 serum and of the conjugates producing optimum bright green fluorescence were used in the subsequent tests. The human acute phase serum in combination with the respective anti-human globulin conjugate did not give any staining of infected cells at working dilutions. The conjugates alone did not exhibit any fluorescence when applied to cover-slip cultures without prior treatment with antisera.

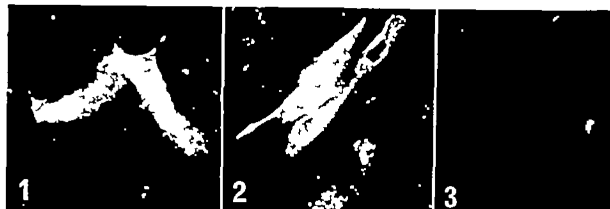


Fig 1 Immunofluorescence (IF) of influenca A virus at the early stage of infection viral antigen, mainly located in the nuclei of infected green monkey kidney (GMK) cells Human anti-influenza A2 serum + sheep anti-human Ig FITC. × 340

Fig 2 IF of influenza A virus at a later stage of infection viral antigen in GMK cells mainly localized in the cytoplasm and concentrated around the nucleus. Human anti-influenza A2 serum + sheep anti-human Ig FITC × 340

Fig 3 Negative IF of GMK cells infected with influenza A virus and stained with human influenza A2 preimmune serum + sheep anti human Ig FITC. × 340

A weak fluorescence seen in RS-virus-infected cells, equal for negative and positive serum, was negligible after absorbing the sera with freeze-thawed cells infected with RS virus. No fluorescence was observed in cells infected with influenza B adenovirus, parainfluenza 1 2 and 3 mumps or measles viruses.

Infectivity Assay in vitro

Influenza A2 Hongkong virus was used which had been isolated from a human case and passaged 7 times in primary monkey kidney cells. The titre, 10^3 $TCID_{50}$ per 0 1 ml was determined after 6 days by means of CPE the occurrence of influenza virus antigen stained by IF and by the haemagglutination activity in the culture medium. Cultures of primary monkey kidney cells on cover slips were inoculated with 0 1 ml of a logarithmically decreasing amount of influenza A, undiluted to 10^{-4} The cultures were incubated for 5 and 18 h and 1 3 and 5 d respectively at 37 C before fixation and staining by IF The virus titre was determined simultanously by testing tenfold dilutions of the virus on cover slips in quadriplicate for each dilution.

RESULTS

Immunofluorescence of Influenza A in Primary Monkey Kidney Cells

The indirect IF pattern of influenza A virus antigen in monkey kidney cells was similar whether the anti human Ig conjugate or the anti human IgG conjugate was used at the working dilution. The anti human Ig conjugate was used regularly on infected monkey kidney cells in this investigation.

The intracellular distribution of antigen varied Some cells mainly showed a fine or coarse granular nuclear fluorescence (Fig. 1) Other cells lacked nuclear fluorescence but showed cytoplasmic fluorescence, occasionally concentrated around the nucleus (Fig 2, 3) Some cells displayed both nuclear and cytoplasmic fluorescence

Time-dose Relationship in an Infectivity Assay

After 5 h only those cover slip cultures which had been inoculated with undiluted virus, displayed positive IF for influenza A. Only nuclear fluorescence was seen. After 10 h cover slip cultures inoculated with 10^3 $TCID_{50}$ or more were positive, and both nuclear and cytoplasmic fluorescence of influenza A were seen. At 18 h incubation with 10 $TCID_{50}$ of virus or more all cover slip cultures were positive for influenza A virus antigen, appearing in single cells and in small plaques.

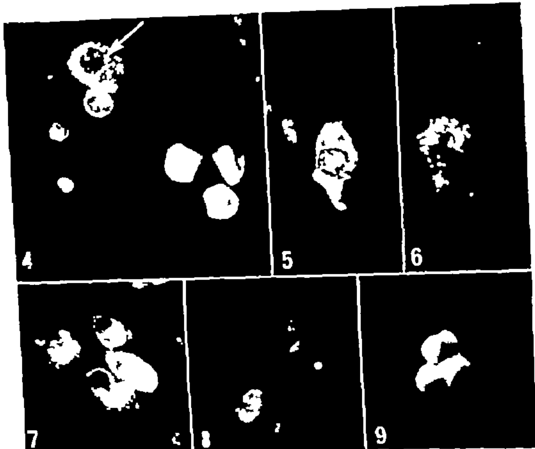


Fig. 4 IF of epithelial cells from nasopharyngeal secretion (NPS) infected with influenza A irus. Arrow denotes a cell with only cytoplasmic, iral antigen I the lower right corner 3 cells with less intensely stained nuclear and cytoplasmic iral tigen. Human anti-influenza A2 serum + sheep anti-human IgG FITC × 650

Fig. 5 An ep thelial cell from NPS containing influenza A irus antigen in the ucleus and I the cytoplasm. A dark halo is seen around the fluorescent part of the nucleus Human anti-influenza A2 serum + sheep anti-human IgG FITC × 750.

Fig. 6 Non-virus-specific inclusions in the cytoplasm of large monocytic cell from NPS stained with sheep anti-human Ig FITC × 650.

Fig. 7 Non-virus-specific IF of "lump of granular material in the cytoplasm of epithelial cells from NPS from patient infected with influenza A rus. Sheep anti-human Ig FITC. × 520

Fig. 8 Epithelial cells from NPS from patient during infection with influenza A irus. The ells are not tained with sheep anti-human IgG FITC × 470

Fig. 9 The same spec men as in figure 8 stained with sheep anti-human IgA FITC in second step Note the ppearance of fluorescent "lump between the nucleus and the cell surface. × 470.

Differ t Type f Immunofluor scence Cells f om NPS

There was bright green fluorescence of in fluenza A in cells from NPS (Fig 4 and 5) Only intact epithelial cells were encountered in the investigation. When the human anti-influenza A2 serum was used in combination with the monospecific anti-human IgG con-

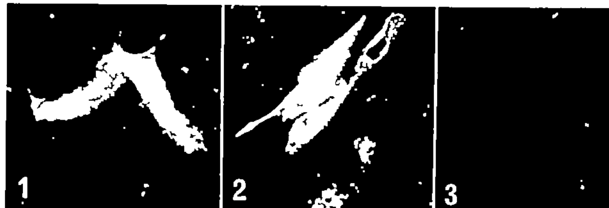


Fig 1 Immunofluorescence (IF) of influenca A virus at the early stage of infection viral antigen, mainly located in the nuclei of infected green monkey kidney (GMK) cells. Human anti-influenza A2 serum + sheep anti-human Ig FITC. × 340

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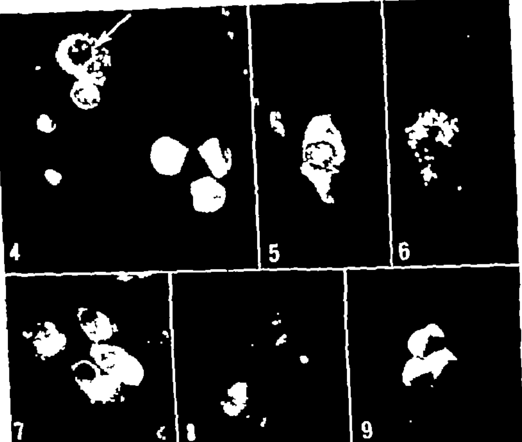


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Different Types of Immunofluorescent Cells from NPS

There was bright green fluorescence of influenza A in cells from NPS (Fig. 4 and 5). Only intact epithelial cells were encountered in the investigation. When the human anti influenza A2 serum was used in combination with the monospecific anti-human IgG con-

jugate, the fluorescence had the same general pattern and distribution as that seen in the primary monkey kidney cells. The acute phase serum combined with the monospecific anti-human IgG conjugate or the conjugate alone did not give any non specific staining in the epithelial cells.

TABLE 1 *Influenza A Infection Detected by Serology by Indirect Immunofluorescence (IF) on Cell Spreads from Nasopharyngeal Secretions (NPS) by IF on Short-term Cover slip Cultures Infected by NPS and by Conventional Virus Isolation from NPS*

No of individuals with positive or negative serology	No of individuals with IF positive cell spreads of NPS/ total no. examined	No of individuals with IF positive cells in short term culture of NPS/ total no examined	No. of individuals with positive specimens of NPS in conventional virus isolation / total no. examined
32 pos [b]	27/32	13/15	6/7
8 neg	0/8	0/3	0/1

Isolation of virus in primary monkey kidney cells followed by haemagglutination and haemagglutination inhibition test.

[b] Fourfold or greater rise of CF antibodies in acute and convalescent phase sera.

However when conjugate of anti human Ig was used instead of monovalent, class-specific anti IgG a non specific fluorescence appeared in addition to the specific influenza virus fluorescence in some epithelial cells, both when the conjugate was applied after pre treatment with the negative or positive influenza A anti sera and when it was used alone This non specific fluorescence could be distinguished from specific influenza fluorescence by differences in localization in the cell and in the fine structure The non specific fluorescence had a coarse granular structure and was mainly localized like a lump" between the nucleus and the cell surface (Fig 7) Furthermore, it was located within the glycoprotein rich part of the cell judging from staining with PAS A similar fluorescence appeared in cell deposits of NPS from patients with other respiratory infections and in specimens from individuals with no signs of infection.

When specimens were stained only with a monospecific, anti human IgA conjugate, the lump" of granular fluorescence appeared to be quite similar to that seen with the anti-human Ig FITC.

Specimens stained for influenza A virus antigen with antiviral sera and monospecific anti human IgG conjugate also exhibited the non-influenza virus specific fluorescence after addition of anti human IgA conjugate (Fig. 8 9) The same was true for the control specimens.

Moreover a blocking test, using unlabelled anti human IgA (sheep anti human IgA for immunodiffusion, Wellcome) before application of anti human IgA conjugate abrogated the non-specific fluorescence almost completely

Large macrophages also contained smooth, round cytoplasmic inclusions, which exhibited fluorescence with the anti human Ig and anti human IgG conjugate alone (Fig. 6) and was accordingly not specific for influenza A virus antigen.

Clinical Material

The results of serology and IF on cell deposits of NPS from 40 patients with the clinical diagnosis of influenza are summarized in Table 1 together with the results of IF of NPS in tissue cultures and the results of conventional virus isolation.

Thirty two of 40 patients had a significant rise of CF antibodies against influenza A. Of the remaining 8 cases one patient with an RS virus infection two with an adenovirus, one with a mycoplasma pneumoniae infection and one with an ECHO virus type 14 infection were diagnosed serologically Another patient had septicaemia and two cases received the diagnosis respiratory infection of unknown aetiology

IF positive cell deposits from NPS were obtained from 27 of 32 patients with serologically diagnosed influenza A infection (84 per cent) In contrast none of the sero-negative

cases displayed any positive IF. Twenty-four of the 32 serologically positive specimens were sampled 1–2 days after the onset of symptoms and the remaining 8 specimens after 3–4 days. In every case two slides were examined with influenza-positive serum and one with the negative serum. The IF showed a predominance of specimens (17 of 23) with easily detectable positive cells (> 5 per slide) 1–2 days after the onset of symptoms. The corresponding figures after 3–4 days of illness were 1 out of 7 specimens. Two specimens were insufficient (too few cells) for examination.

Monkey kidney cells in cover slip cultures inoculated with specimens from serologically positive subjects displayed a virus-specific IF in 13 of 15 cases (87 per cent). The two negative cases were also negative by IF on NPS, and one examined was also negative by conventional virus isolation.

DISCUSSION

This investigation has demonstrated the value of indirect immunofluorescence (IF) using human convalescent serum for rapid identification of influenza A in cell spreads of nasopharyngeal secretions (NPS). The reliability of this method was borne out from various observations. Firstly there was good agreement between IF positive cells in cell spreads of NPS and IF positive monkey kidney cells in short term cultures inoculated with NPS. Secondly after proper absorption, the human influenza A sera from acute and convalescent phases did not give rise to any cross reactions with a panel of heterologous virus antigens.

While polyvalent anti-human Ig conjugate was satisfactorily specific for the IF test on infected cells in tissue cultures, it occasionally caused a non-virus-specific IF in cell spreads from NPS with a pattern different from that of influenza A. It appeared even after staining with the anti-Ig conjugate alone and was seen in specimens of a variety of respiratory infections, as well as in specimens from individuals with no symptoms from the respiratory tract.

However this error could be avoided by using a monospecific, sheep anti-human IgG conjugate which gave specific staining of the influenza A virus antigen within the epithelial cells of NPS, judging both from the negative controls and the negative results of staining with the conjugate alone.

Thus, anti-human IgG conjugate should be used for indirect IF on virus infected cells from NPS.

The non-virus-specific IF was apparently caused by anti-IgA antibodies in the polyvalent, anti-human Ig conjugate bound to IgA in the epithelial cells. This was borne out from our observation that the non-virus-specific IF appeared when a mono-specific anti human IgA was applied to the epithelial cells in a third step after the anti-human IgG conjugate in the direct IF test for influenza A. It also appeared after staining with the anti IgA conjugate alone and was blocked by the previous application of unlabelled, anti-human IgA serum. Thus, our investigation, based on cellular material, indicates the presence of IgA in the epithelial cells of the nasopharynx. These findings agree with those of recent studies on biopsy material from different mucous membranes (1) which showed by double-tracing by IF that not only the secretory component of IgA but also IgA appears in epithelial cells.

In the clinical study the diagnosis of influenza A was settled by IF applied directly on cell deposits from NPS in 84 per cent of serologically positive influenza A patients. These results agree with other similar investigations (2, 4, 5, 6, 8, 9). In our study specimens taken within two days after onset of symptoms seemed to be readily diagnosed by this method. In contrast to some other investigators (11, 12, 13) we did not record any positive results of IF applied to cell spreads from NPS without positive influenza serology.

Furthermore, a comparable diagnostic yield (87 per cent) was achieved when specimens of NPS were incubated in cultures of monkey kidney cells for 18–66 h, and the virus subsequently was identified as influenza A by indirect IF. We found a comparable sensitivity of this method during an outbreak of influ-

enza A in 1969 (*Olding-Stenkvist & Grandien* unpublished data) when tissue cultures incubated with throat washings and autopsy material from the respiratory tract of 50 individuals were tested for influenza A virus by indirect IF and compared with conventional virus isolation. Eighteen of 20 specimens (90 per cent) positive for influenza A in conventional virus isolation tests were positive in the IF test following short term incubation

The infectivity assay performed *in vitro* showed that even after the inoculation of small doses of influenza A (10 $TCID_{50}$) into cell cultures on cover slips, the virus antigen was easily detected by IF after only 18 hours. Those results indicate that the method is sensitive enough for rapid diagnosis, even when the specimens contain only small quantities of virus.

In conclusion, the sensitivity and reliability of the two IF methods presented for rapid diagnosis of influenza A seemed to be comparable. When sampling of proper material is possible, IF directly applied to cell spreads from the upper respiratory tract may be preferable, thereby reducing the time for diagnosis from 1–3 days to 3–4 hours.

The incompleteness of the clinical diagnosis of influenza and the need for laboratory tests is illustrated by this study. All patients fulfilled the clinical criteria for influenza yet only 80 per cent of the patients were confirmed as cases of influenza A infection by laboratory tests.

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STUDIES ON MYCOBACTERIA ISOLATED FROM ANIMALS, WITH SPECIAL REFERENCE TO THE AGGLUTINATION TEST

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Saitanu, K. Studies on mycobacteria isolated from animals, with special reference to the agglutination test. Acta path. microbiol. scand. Sect. B, 85: 303–307, 1977.

Ninety-three strains of slowly-growing mycobacteria were studied biochemically. Ninety of these were isolated from animals (pigs, cattle, dog and poultry) and three from dust and sawdust-bedding in a pighouse. One strain from a lymph node of a pig was identified as *M. gordonae*. Ninety-two strains fitted into the *M. avium-intracellulare* complex. Of the 92 biochemically confirmed *M. avium-intracellulare* strains, 78 were tested serologically *ad modum Schaefer*. Of 73 strains from pigs, one was serotype 1, fifty serotype 2 and eight serotype 8, while two could not be typed and twelve were autoagglutinable. Three strains from pighouse environment were serotype 8 and two from cattle and a dog were both serotype 2. A slight modification of *Schaefer's* agglutination method, using smaller amounts of antigen and antiserum, was developed.

Key words: Mycobacteria; animals; agglutination.

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Received 21.i.77. Accepted 21.ii.77

In their comprehensive study of coincidental outbreaks of tuberculosis among poultry and swine in an Oregon farm, *Mohler & Washburn* (1908) presented convincing evidence of tuberculous poultry as an important source of tuberculosis in swine. Based on similar observations of his own *R. Rasmussen*, practising veterinarian in Faaborg, Denmark, reopened the topic in 1911 and the problem of "avian tuberculosis" in mammals naturally became one of the research priorities of the Danish campaign against tuberculosis in animals. The rôle of avian mycobacteria in the epidemiology of tuberculosis in cattle, swine and other domestic animals needed clarification, as did also the interference of sensitization by avian bacilli with diagnostic tuberculin testing programmes. The works of *Bang* (1913, 1929) and *Plum* (1925) created the scientific basis upon which practical solutions to these problems could be instituted.

With the eradication of *Mycobacterium bovis* and a concurrent decrease in avian tuberculosis largely due to structural changes in poultry husbandry, the frequency of exposure of other animals, especially swine, to tuberculous infection decreased drastically. However, meat inspection services reported repeatedly the finding of tuberculosis in swine,

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while epidemiological investigations failed to locate either bovine, avian or human sources of infection This situation logically drew the attention of research to other agents which might produce tuberculosis-like lesions, e.g *Corynebacterium equi* (*Holt & Amundsen* 1936 *Bendixen & Jepsen* 1938) However the important clue to a better understanding of the epidemiology of mycobacterial infections came only later Newer developments in methodology for the classification of mycobacteria have resulted in a broader insight into some classical taxonomic entities and deprived a number of new species of their former anonymity

MATERIAL AND METHODS

While examining an epizootic of *Mycobacterium intracellulare* infection in Danish pigs (*Saitanu & Holmgaard* 1977) the author studied biochemical and serological classification of animal strains of mycobacteria. For origin of the strains, see Table 1

Biochemical classification The following characters were determined Growth rate at 37 C (Löwenstein-Jensen medium) scotochromogenicity catalase (*Kubica et al.* 1966) Tween 80 hydrolysis (*Wayne et al.* 1964) nitrate reduction (*Virtanen* 1960) tellurite reduction (*Kilburn et al.* 1969) arylsulphatase (3 days and 2 weeks) (*Whitehead et al* 1953) amidase tests (*Bönicke* 1962)

Serological classification The serological test system used in this work is the agglutination method introduced by *Schaefer* in 1965 For preparation of our antigens and antisera two reference strains for each of 25 *M avium-intracellulare* serotypes and corresponding antisera were placed at the disposal of the author by *W B Schaefer* The National Jewish Hospital and Research Center Denver Colorado, U.S.A and *The U.S./Japan Cooperative Medical Research Programme NIAID*

In setting up the serological system and in the actual testing procedures, *Schaefer's* original description from 1965 was followed, supplemented by his personal advice (1974) The Schaefer method requires each strain to be tested against 2 x 25 antisera diluted to the reciprocal of the titre. Equal volumes of bacterial suspension and diluted antiserum are added to 2 x 25 agglutination tubes, plus one control Reading is made after three hours at 37 C, and again after incubation over night A test strain is considered type-specific if agglutinated by both antisera of one type and not by both antisera of any other type If a strain is agglutinated by one antiserum of one type only or by two antisera of two serotypes, the agglutinin-absorption test is applied. The bacterial suspensions are harvested from Roux flasks cultures of Middlebrook 7H10 medium supplemented by Middlebrook OADC enrichment (Difco) Incubation is made for 2–3 weeks at 37 C or as suggested by *Schaefer* (1974) at 30 C to improve stability of suspensions. The bacterial growth is washed off and suspended in 0.15 M phosphate-0.8 per cent saline buffer containing 0.5 per cent phenol The suspension is kept at room temperature for seven days to inactivate the living organisms, then centrifuged and washed with the phosphate-saline buffer until the supernatant is clear The density of the final suspension is measured spectrophotometrically at 525 nm in 20 mm tubes and adjusted to absorbance 0.3

At least 2 x 25 x 0.5 = 25 ml of antigen is needed for each strain to be typed. To avoid the difficulty of having to work with such large volumes of antigen of slowly-growing mycobacteria, the author developed a slightly modified Schaefer method, using only 0.25 ml of antigen and antiserum per tube and reducing the density of the antigen from 0.3 to 0.15

RESULTS

Cultural and biochemical tests applied to the 90 animal strains of mycobacteria, and to 3 isolates from animal environment as listed in

TABLE 1 *Number and Origin of Strains*

Pig	Pighouse)	Poultry	Cattle	Dog	Received from
76					K. Lautrup Nielsen
			1		N Skovgaard
8	3	4		1	Own isolates
84	3	4	1	1	Total 93

) *Dust and sawdust bedding from pig pens.*

Table 1 showed that 92 strains fitted into the *M. avium-intracellulare* complex, whereas one strain, isolated from tuberculous-like lesions in lymph nodes of a pig was identified as *M. gordonae*. Table 2 gives a summary of the results of the tests.

Time limits permitted 78 strains of the *M. avium-intracellulare* complex to be submitted to serotyping *ad modum Schaefer*. Twelve strains proved to be autoagglutinable and two strains could not be typed by the system used. Of the remaining strains, one was serotype 1, 52 serotype 2 and 11 serotype 8 (Table 3). The serotype 8 strains originated from four swine herds involved in the same epizootic, whereas the serotype 1 and 2 strains represent a large number of unrelated individual cases.

TABLE 2. *Characteristics of* M. gordonae *and* M. avium-intracellulare

	M. gordonae (1 strain)	*M. avium-intracell.* (92 strains)
Growth rate more than 7 days	+	+
Scotochromogenicity	+	—
High catalase	+	—
Tween 80 hydrolysis (14 days)	+	—
Nitrate reduction	—	—
Tellurite reduction	+	+
Arylsulphatase 3 days	—	—
2 weeks	+	±
Acetamide	—	—
Benzamide	—	—)
Isonicotinamide	—	—
Nicotinamide	—	+
Pyrazinamide	—	+
Salicylamide	—	—
Allantoin	—	—
Succinamide	—	—
Malonamide	—	—
Urea	—	—

— = 0–19 % pos. ∓ = 20–49 % pos.
± = 50–79 % pos. + = 80–100 % pos.

) Results of the amidase tests are based on 62 strains.

TABLE 3. *Serological Typing* ad modum Schaefer *of 78 Strains of* M. avium-intracellulare

Serotype	Pigs	Pighouse	Cattle	Dog	Total
1	1				1
2	50		1	1	52
8	8	3			11
Non-typable	2				2
Autoagglutinable	12				12
Total					78

DISCUSSION

Since 1945–50 the elimination of *M. bovis* from its bovine host has left avian and human reservoirs as the only source of tuberculosis of mammals in Denmark. Most mammals are susceptible to both *M. avium* and *M. tuberculosis* but, with a few exceptions, their virulence to mammals is much lower than that of *M. bovis*. The infection generally runs the course of a self-limiting disease because of the proliferative rather than destructive features of the pathological lesions produced by these heterologous agents in most animal species. Thus animal tuberculosis today has lost most of its devastating effects on animal health. Meat inspection statistics show a frequency of tuberculous infection in slaughtered pigs of 1.5 per 1000, these being mostly localized primary lesions in cervival or mesenteric lymph nodes. Similar localized lesions are rarely found in cattle. In accordance with veterinary legislation the finding of tuberculosis in cattle, horses and pigs must be reported, and in many instances diagnosis must be confirmed by laboratory examination, including cultural type determination. *M. avium* is the predominant agent found in such material.

In 1968 *Engbæk et al.* published a series of studies of *M. avium* isolated from animals and man in Denmark. The animal strains, collected in 1961–63, were mainly from pigs, cattle and poultry. A selection of 41 animal strains were serotyped by *Schaefer*. One strain was classified as serotype 1, 34 as serotype 2, four could not be classified, and two were

aberrant strains. Among 22 human isolates, one was serotype 1 nine serotype 2 and seven serotype 8 Davis (*M intracellulare* serotype 8) The remaining five strains represent various other types.

The present work confirms the predominant rôle of *M avium* serotype 2 in animal infections. However in addition it has demonstrated that *M intracellulare* serotype 8, found previously in humans, also occurs in pigs in this country Similar findings have been reported from several other countries e.g., Japan U.S.A. and Canada (*Yachida & Shimizu 1973 Mitchell et al 1975 Thoen et al 1975 a Simon 1976*) The *M intracellulare* serotype 8 epizootic examined by *Saitanu & Holmgaard 1977* comprising about 800 cases, originated from a single breeding centre with 200–250 sows. Apparently the piglets had caught the infection from environmental sources while living with their mothers during the suckling period. In modern large scale pig farming contacts between pigs and poultry are practically non-existent.

For all practical purposes a prevalence of 1.5 per 1000 of tuberculosis-like lesions in lymph nodes of pigs is an insignificant figure. However, this figure has remained constant in Denmark year by year since 1969 From a scientific point of view it is still an interesting question as to the source of infection for these 15 000 animals per year and what kind of infection is involved. The answers might perhaps throw some light also on the epidemiology of the rare cases of human infection caused by the same organisms. In efforts to find a solution, a reliable system for typing the epidemic strains would naturally be of great help

The Schaefer agglutination method requires each strain to be tested against a double set of antisera each type represented by two reference strains. Taking into account the close antigenic relationship of the complex as a whole, one might well doubt the validity of using unabsorbed sera. The sera are used in dilution to the reciprocal of the titre. As regards our sera, this was equal to 160–640 in most cases 320 with only four exceptions. None of the strains tested was agglutinated by two antisera of two types. Cross reaction showing partial agglutination at the second reading with one of the two heterologous antisera, occurred in strains agglutinated by both homologous sera. The typing of four strains (nos. 358 from pig and 359 360a, 360b from sawdust-bedding and dust) was confirmed by agglutinin-absorption tests, since only one antiserum type 8 reacted with the strains. In our material, the main cause of failure of identification was autoagglutination. Other serological techniques could presumedly be applied to unstable strains.

The preparation of sufficient quantities of antigen from slowly-growing mycobacteria is a laborious task. Several workers have tried to cut down the expenditure of antigen and antisera (*Reznikov & Leggo* 1972 *Thoen et al 1975 b*) The author likewise has tried a modification to save antigen and serum. This modified Schaefer technique, without any change of principle has been found to be equally useful

The finding on one occasion of *M gordonae* from a tuberculosis-like lesion in lymphatic tissue cannot be evaluated further It may be mentioned that *Viallier et al* (1963) isolated the organism from tuberculous-like lesions of bovine teats, and *Shimizu & Tsukamura* (1974) found *M gordonae*-like organisms in skin lesions of the bovine udder

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dicate that such properties might have a decisive influence on the host-parasite interaction (22, 27–30). This investigation was initiated to examine whether a connection could be established between general physico-chemical characteristics of the bacterial surface such as charge and hydrophobicity and association with the intestinal mucosa. Although enterobacteria are normally a minor part of the intestinal flora, such bacteria were chosen as models, since they often appear as intestinal pathogens and have been thoroughly investigated in relation to other cells and cell systems (17, 27).

MATERIALS AND METHODS

Strains. The smooth (S) phagocytosis-resistant *Salmonella typhimurium* 395 MS and the rough (R) phagocytosis-sensitive, glucosyl-I-transferaseless mutant MR10 derived from it has been described in detail with respect to chemical composition (15), phage pattern (19), virulence (8), phagocytosis (27) and physico-chemical surface characteristics such as charge and liability to hydrophobic interaction (4). Two *Escherichia coli* O antigen test strains Cu 4414/14 and Stoke W representing rough (O 14:K7 chemotype I) and smooth (O 111:K58 chemotype X) strain respectively were kindly supplied by Dr. *F. Ørskov*, Copenhagen.

Cultivation. All strains were kept on agar slants at 4 C before use. The bacteria were grown in 10 ml of nutrient broth (Difco) at 37° C for 18 h. The bacteria were harvested by centrifugation (6000 × g) and resuspended in Krebs-Ringer phosphate buffer with 10 mM glucose, pH 7.2 (KRG). In most experiments the bacteria were killed at 56 C for 1 h and washed twice in phosphate-buffered saline, pH 7.2 (PBS). The concentrations of viable and heat-killed bacteria were estimated by a Turner spectrophotometer (650 nm).

Labelling. Two different isotopes, ^{51}Cr and ^{125}I, were used in order to be able to perform concurrent tests on two bacteria. Labelling of heat-killed bacteria with ^{51}Cr and ^{125}I was performed as described previously (28). An Auto-gamma-scintillation counter with an NaI-crystal (Packard Instrument, Downers Grove, Ill. U.S.A.) was used for radioactivity determinations.

Enzyme assays. N-acetyl-beta-glucosaminidase activity assayed using p-nitrophenyl-N-acetyl-beta-D-glucosaminide as substrate (23) and the extraction measured as E_{410} (2, 3). Triton X-100 (0.1 per cent) was used to dissolve particle-bound enzyme.

Preparation of intestinal segments. Female Sprague-Dawley mice weighing 18–22 grams were used. The animals were fasted for 18 h before being anaesthetized in a glass jar containing a cotton wool pad soaked with ether. The abdomen was opened in the midline, and the intestine cut at the duodenojejunal flexure and the ileocaecal junction. A 2.0 × 80 injection needle was inserted into the jejunum, and the lumen of the intestinal segment in between the cuts was rinsed by gentle flushing with KRG using a 5 ml syringe connected to the needle.

In early experiments, the mice were killed by cervical dislocation instead of using anaesthesia and the intestine was removed before rinsing, but, except for this variation, the segments were handled in the same way as described below. Sections for morphological examination were stained with haematoxylin-eosin (HE) and periodic-acid-Schiff (PAS).

Association test. Immediately after rinsing, the intestinal segment was excised and mounted on two injection needles 2.0 × 80 (sharp ends of needles smoothed). The needles were fixed at the ends of a plastic bath with the segment in between. The bath contained KRG (pH 7.4, 37 C) and was oxygenated continuously.

Four segments could be run at a time. The term association is used to cover attachment and possible endocytosis.

Pumping of heat-killed radioactively-labelled bacteria through a segment of small intestine. 1) Small volume pumped rapidly. The radioactivity of the heat-killed and labelled bacteria was measured and bacteria were taken to obtain an activity of 10⁵–10⁶ cpm for ^{125}I and 30,000–100,000 for ^{51}Cr. These radioactivities represented approximately the same number of bacteria (0.5–4.0 × 10⁸ bact.). One strain labelled with ^{125}I and another labelled with ^{51}Cr were mixed, and KRG was added to 0.4–1.0 ml. The suspension was injected into the plastic tubing close to the intestinal segment and pumped through the segment at 2.0 ml/min with a 6-channel peristaltic pump (Ole Dich, Copenhagen, Denmark). The pumping was continued for 45–60 min. Oxygenated KRG without bacteria was pumped 5–10 min before and 25–40 min after the bacteria had been added to the tube.

The material voided at the end of the intestinal segment was collected in portions every 5 min for 45–60 min and the radioactivity determined. The last sample never exceeded 5000 cpm for ^{125}I or 1000 cpm for ^{51}Cr. The intestinal segments were then removed, each cut into four pieces of equal length and dipped several times into KRG. Very little radioactivity came off during that process. The radioactivity was measured and the association of bacteria expressed as

$$\frac{\text{segment (sum of the four pieces)}}{\text{segment + voided}}$$

Acta path. microbiol. scand. Sect. B, 85 308–316, 1977

ASSOCIATION OF SOME ENTEROBACTERIA WITH THE INTESTINAL MUCOSA OF MOUSE IN RELATION TO THEIR PARTITION IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

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Perers, L., Andåker, L., Edebo, L., Stendahl, O. & Tagesson, C. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta path. microbiol. scand. Sect. B 85 308–316, 1977.

The association of enterobacteria with mouse intestinal mucosa has been investigated by pumping heat killed, radioactively-labelled bacteria through the gut lumen *in vitro*. Approximately 20 cm of the small intestine proximal to the ileo-caecal valve was rinsed, excised and maintained in an organ bath. By using two different bacteria labelled with different radioactive isotopes, the relative association of the two bacteria pumped through the same piece of gut was determined. Cross-labelling showed that choice of isotope did not affect the association. *Salmonella typhimurium* 395 MR10 was used as reference and the other bacteria investigated related to it. *S. typhimurium* MR10 and Escherichia coli O 14 K7, which are relatively lipophilic, showed greater association than *S. typhimurium* 395 MS and *E. coli* O 111 K58, which are more hydrophilic. Prolonged incubation of bacteria with the length of intestine *in vitro* leading to damage of the brush border of the mucosal epithelium enhanced the association of the bacteria. These data suggest that similar physico-chemical surface properies govern the association of certain enterobacteria to the intestinal mucosa as in phagocytosis with professional phagocytes.

Key words: Enterobacteria; intestinal mucosa; association; aqueous polymer two-phase system; mouse.

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Received 15.iii.77 Accepted 25.iv.77

The host microbial relationship in the intestinal tract and the characteristics of commensal and pathogenic bacteria to explain their biological role are largely unknown. Since the contents of the gut are propulsed towards defecation, association of bacteria with the intestinal wall would influence the population dynamics of the bacteria and probably also their relation to the host intestinal mucosa (14, 25). Accumulating data on the chemical composition of the cell surface of enterobacteria and their physicochemical properties (6, 20, 21, 28, 29) in-

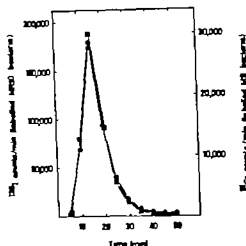


Fig 1 The elution profile of ^{51}Cr-labelled *S typhimurium* 395 MS (●) and ^{125}I-labelled MR10 (■) pumped through the isolated intestine at rate of 0 2 ml/min. One ml fractions were collected

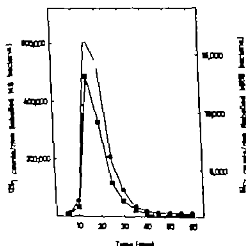


Fig 2 The elution profile of ^{125}I-labelled *S typhimurium* 395 MS (●) and ^{51}Cr-labelled MR10 (■) pumped through the isolated intestine at rate of 0 2 ml/min. One ml fractions were collected

collection of the voided fluid showed a peak of radioactivity in the 10–15 min fraction (Figs 1–2) The relative activity of *S typhimurium* 395 MS and R10 cells in the different fractions collected throughout the experiment remained unaltered. This was observed both when MS was labelled with ^{51}Cr and R10 with ^{125}I (Fig 1) and *vice versa* (Fig 2) These data indicate that labelling with either of the isotopes caused no significant differences. It shows further that bacteria which adhered to the intestinal mucosa, did so rather firmly so that there was little elution of bacteria under the experimental conditions employed

Two typical test protocols corresponding to Figs. 1–2 are shown in Table 1 In both experiments the association of R10 (3.6 and 6.7 per cent) was greater than that of MS (0.64 and 0.2 per cent) A larger series of experiments showed greater variation between individual experiments (Table 2) However the difference between R10 and MS is highly significant ($p < 0.0005$)

Slow Flow of Larger Volume Through a Segment of the Intestine

When 4 ml of radioactively-labelled bacteria was pumped through a similar segment of intestine at 0 1 ml/min, the peak of activity was recovered in the fractions collected after about 50 min, and in some experiments radioctivity was still demonstrated after 80–90 min but not after 100 min (Fig. 3) The proportion of the radioactivity associated with the intestinal segments was for R10 21.8 per cent and for MS 3.9 per cent (Table 3) the difference is significant ($p < 0.0005$)

Association of Viable Bacteria

Viable counts of voided bacteria gave higher relative values for *S typhimurium* 395 MS (90 per cent) than for *S typhimurium* 395 MR10 (40 per cent) These data, which were obtained after slow pumping of a relatively large volume of bacterial suspension, were in accordance with those for heat killed and labelled bacteria. However uncontrolled effects such as killing and multiplication of bacteria influence these results.

All tests were performed with a pair of two different bacteria, one labelled with ^{51}Cr and the other with ^{125}I. In all experiments, *S. typhimurium* R10 was one of the pair and used as reference. R10 was generally labelled with ^{125}I and the other strain with ^{51}Cr. In control experiments, R10 was labelled with ^{51}Cr and the other with ^{125}I to check the influence of labelling on the association with the intestinal wall. The binding of free radioactive compound to the intestinal wall was checked and found to be negligible.

Samples (10 ml) were taken from the bath before and after the run for radioactivity measurement to determine leakage. The activity of the samples was low and did not differ significantly from background counts. Recovery was calculated for all tests as the radioactivity of the sum of pour pieces of the intestinal segment + suspension voided + remainder in the test tube + tubing leading to and from the segment divided by that of the original bacterial suspension. The average recovery in 16 experiments was for ^{125}I 102 per cent ± 1.35 (± S.E.M.) and for ^{51}Cr 95 per cent ± 1.10 (± S.E.M.).

b) Larger volume pumped slowly. In a few experiments the methods described above were used, except that 4 ml bacterial suspension was pumped at 0.1 ml/min and the samples voided were collected every 10 min. In these experiments, which were continued for two hours, no extra oxygen was supplied to the KRG pumped through the gut for maintenance.

Partition in two-phase systems. The two-phase system was made up to contain in total 4.4 per cent (wt/wt) polyethylene glycol and 6.? per cent (wt/wt) dextran in 0.03 M Tris buffer. It was prepared from stock solutions of 20 per cent polyethylene glycol 6000 (Carbowax 6000, Union Carbide, New York, N.Y.), 20 per cent dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden), 0.1 M tris (hydroxymethyl) aminomethane (Tris) buffer pH 7.0 and distilled water and was allowed to equilibrate at 4 C overnight. To analyse surface charge and hydrophobicity substituted (trimethylamino, sulphonate, palmitoyl) PEG was added to the system. For detailed description, see Table 4.

Two millilitres of the bottom phase (dextran-rich) and 2 ml of the top phase (polyethylene glycol rich) were pipetted into test tubes. To each tube was added 0.1 ml of ^{51}Cr labelled bacteria suspended in 0.03 M Tris (? × 10 /ml). The tubes were inverted 20 times for mixing, and the phases were allowed to settle for 20 min at 20 C. Then 0.5 ml samples were withdrawn from each phase, and the radioactivity was measured to determine the percentage of cells in each phase. The standard deviation in five similar experiments was 5 per cent.

RESULTS

Evaluation of the Intestinal Model

Morphology. Since initial experiments showed that mucosal damage resulted in altered association of bacteria, special care was taken to evaluate the model with respect to mucosal integrity. Sections for microscopy were taken from segments which had been perfused for 30, 45, 60 and 75 min. The 30 min section did not differ from that taken at 0 min. At 45 min there was slight to moderate submucous oedema, but the mucosal structure was intact with an uninterrupted surface lining. At 60 min the oedema had increased and in places, single villi showed slight damage of the surface and interrupted PAS-positive surface lining. At 75 min there was partial disintegration of villous structure with severe oedema and moderate to severe cytological changes. After 2 h even more severe mucosal damage was seen, with almost total mucosal disintegration and exposition of the submucosa.

Peristalsis. With a gentle and fast preparation in an anaesthetized animal, the length of intestine showed no or only very slight peristaltic concentrations. If the animal was first killed and the segment removed before running, there was usually moderate to severe peristalsis and the mucosa was disrupted within 35–60 min.

Release of mucosal enzyme. The release of N acetyl beta-glucosaminidase extracellularly was low and constant throughout a 60 min run when the segment showed no or only very slight peristalsis. In experiments where the mucosa ruptured, this was accompanied by a sharp rise in enzyme activity which usually was voided 1–2 fractions before cellular debris was observed macroscopically.

Fast Flow of Small Volume Through a Segment of the Intestine

The pumping of 0.4–1 ml of a mixture of heat killed radioactively labelled *S. typhimurium* 395 MS and R10 through the length of intestine at a flow of 0.2 ml/min and

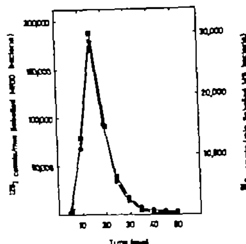


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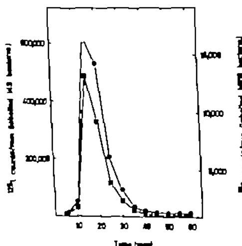


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TABLE 1 *Protocols Showing the Association and Recovery in Two Association Experiments with S. typhimurium MS and MR10 Labelled with ^{51}Cr and ^{125}I Respectively (Exp I) and Vice Versa (Exp. II)*

	Experiment I		Experiment II	
	MS-^{51}Cr	R10 ^{125}I	MS ^{125}I	R10 ^{51}Cr
	(cpm)		(cpm)	
Bacterial suspension before pumping a)	75 160 c)	484 900	1,620,000	33,820
Suspension voided	66,850	421,800	1,637,000	29,090
1st segment of intestine (oral end)	190	9 630	560	418
2nd segment of intestine	100	3,520	1030	480
3th segment of intestine	90	1,550	690	300
4th segment of intestine	50	950	660	400
Tubing leading to the intestine	1 150	46 230	57,925	3,673
Tubing leading from the intestine	590	3 630	8 706	385
Surrounding bath before exp b)	30	130	—	—
Surrounding bath after exp b)	20	90	—	—
Recovery	92 per cent	101 per cent	105 per cent	101 per cent
Association to the whole length of intestine	0 64 per cent	3 6 per cent	0 18 per cent	6 7 per cent
Association to tubing	2.3 per cent	10 4 per cent	3.9 per cent	11.3 per cent

a) A mixture containing 5 × 10^7 (Exp I) or 1 5 × 10^7 (Exp. II) cells of each *S typhimurium* 395 MS and MR10

b) 10 ml of oxygenated KRG

c) cpm.

TABLE 2 *Association of* S typhimurium *395 MS and MR10 to Mouse Intestinal Mucosa* a)

Strain	Number of experiment	Association (per cent) b)	Recovery (per cent) b)
S typhimurium 395 MR10	16	3.90 ± 0.53	102 ± 1.35
S typhimurium 395 MS	9	1 03 ± 0 12	93 ± 1 49

a) One ml bacterial suspension pumped at a rate of 0.2 ml/min. Most bacteria voided within 30 min.

b) Mean value (M) ± Standard error of the mean (S.E.M.)

TABLE 3 *Association of* S typhimurium *395 MS and MR10 to Mouse Intestinal Mucosa* a)

Strain	Number of experiment	Association (per cent) b)	Recovery (per cent) b)
a) Four ml bacterial suspension pumped at a rate of 0 1 ml/min Most bacteria voided within 90 100			
S typhimurium 395 MR10	3	21.8 ± 7.5	84 ± 3.2
S typhimurium 395 MS	3	3 9 ± 1 4	84 ± 3.8

a) Four ml bacterial suspension pumped at a rate of 0.1 ml/min. Most bacteria voided within 90–100 min

b) Mean value (M) ± S.E.M

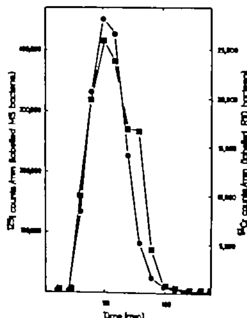


Fig 3 The elution profile of ^{125}I-labelled *S typhimurium* 395 MS (●) and ^{51}Cr-labelled MR10 (■) pumped through the isolated intestine at rate of 0 1 ml/min. One ml fractions were collected.

Association to Mouse Intestinal Mucosa of E. coli *O 14 and* E. coli *O 111 and Partition in Two-phase Systems*

In order to reduce the influence of chance variations between different experiments, the association of *S typhimurium* 395 MS and of *E. coli* Su 4414/41 (O 14:K7) and *E. coli* Stoke W (O 111:K58) was calculated as per centage of that of the concurrently run *S typhimurium* 395 MR10 (Table 4) The association of *S typhimurium* 395 MS was 27 per cent of that of MR10 The association of *E coli* O 14 was 65 per cent of that of R10 whereas the association of *E. coli* O 111 was 18 per cent (Table 4)

Partition of *E coli* O 14 in the two-phase systems resembled that of MR10 and the bacteria showed tendency to interact with positively charged PEG and with the palmitoyl side chain of covalently substituted PEG In contrast, *E. coli* O 111 accumulated into the PEG-rich top phase and was not influenced by charged or palmitoyl-substituted PEG (Table 4)

DISCUSSION

In the host-enterobacterial relationship in the intestinal tract, contact is established between he mucosal surface and the bacterial lipopolysaccharide (LPS) or other surface structures. For *Escherichia coli* (1 4 7 26) *Salmonella* (11 12, 31) and *Shigella* (18) this contact has been shown morphologically to take place at the brush border mainly at the upper parts of the intestinal villi. Then

TABLE 4 *Partition in Two-phase Systems with Charged or Hydrophobic Polymers and Association to Mouse Intestinal Mucosa of Two* E. coli *Strains and S typhimurium 395 MS and MR10*

Strain	Chemotype	Per cent bacteria in top (T) and bottom (B) phase								Association d)
		positively a) charged PEG		uncharged PEG		negatively b) charged PEG		hydrophobic c) PEG		
		T	B	T	B	T	B	T	B	% of MR10
S typhimurium MS	Group B	95	5	95	5	95	5	96	6	27 ± 2.6
S typhimurium MR10	Rd-mutant	50	25	9	75	10	80	50	20	100
E coli 014	I	49	26	8	62	5	70	71	8	65 ± 6.0
E. coli 0111	X	95	6	97	7	95	6	97	8	18 ± 4.2

a) 6.25 per cent of PEG was substituted with Trimethylammo (TMA) PEG
b) 6 25 per cent of PEG was substituted with Sulphonate (S) PEG
) 0 1 ml of 2 per cent palmitoyl-PEG was added to the uncharged system.
d) One ml bacterial suspension was pumped at 0 2 ml/min.
Mean alue ± S.E.M

some bacteria are found within the mucosal epithelium (18 26 32 33) However the factors governing the association with and endocytosis in the intestinal epithelium have been only sparsely investigated.

Preparations of small intestine for *in vitro* analysis have been used extensively for the study of intestinal absorption (23) Using a modification of the technique described by *Fisher & Parsons* (10) we observed an almost intact mucosal lining after 60 min but with the presence of moderate submucous oedema similar to their results. We found peristalsis to be a poor criterion of viability as reported earlier (10 16) Strong regular peristaltic contractions often occurred after a poor preparation and preceded disruption of the mucosa in 35–60 min. In prolonged experiments with slow flow of large volume through the intestine, strong peristalsis was the rule, and histology of these segments after two hours showed a damaged mucosa with exposition of the submucosa In the latter type of experiments the association of e. g R10 was much higher (21 8 per cent) (Table 3) than when care was taken to avoid damage of the mucosal lining (3.90 per cent) (Table 2) thus indicating that the intact brush border prevents attachment of bacteria This might also be a mechanism for the preferential attachment of enterobacteria at the tips of the villi where the epithelium is being degenerated and shed Heat killed bacteria have been used in these experiments, since they permit study of the association with the animal cells without the influence of killing or multiplication of the bacteria. Heat killed bacteria have been used previously in the analysis of those surface properties of bacteria which influence phagocytosis and virulence (27) Such heat killed and radioactively labelled *Salmonella* bacteria behave identical to viable bacteria in a two-polymer phase system aimed at analyzing the cell surface (28) This does not exclude that mechanisms essential for the invasion *in vivo* are missing in heat killed bacteria e. g activities for derangement of the brush border That this may be the case is indicated by the increase in association of live *Salmonella* bacteria to HeLa cells (17) and to a spontaneously degenerated mucosa.

The association of the heat killed enterobacteria to mouse intestinal mucosa differed conspicuously between the different strains employed. The association was greatest for *S typhimurium* 395 MR10 and *E. coli* Su 4411/41 (O 14 K7) (Table 4) Both MR10 and O 14 have the R type LPS MR10 is a Rd mutant with the terminal sugar L-glycero-D-manno-heptose (heptose) and O 14 has a complete core type of LPS (20) Both these bacteria showed negative charge and a tendency to hydrophobic interaction in the two-phase systems. In contrast, *S typhimurium* 395 MS and *E coli* Stoke W (O 111.K58) which associated less to the mucosa contain S-specific sugars in their LPS (20) and showed neither hydrophobic interaction nor charge This indicates that general physicochemical properties of the bacterial surface such as charge and hydrophobicity play a role in determining the association with the mucosa

In several other systems involving association of bacteria with cell membranes, similar surface characteristics govern the outcome of the interaction. Rough lipophilic *Salmonella* mutants and their LPS are readily phagocytosed by granulocytes in contrast to the smooth ones (27) In short term experiments focusing on the association of S and R strains of *Salmonella* to HeLa cells (17) the rough bacteria attached and were internalised to a greater extent than the smooth strain. In contrast to interaction with professional phagocyes, this process required living bacteria. Furthermore, adsorption of *S Senftenberg* to slices of rabbit ileum was in the same range as that of *V cholerae* (11) However the adsorption of *S senftenberg* was strikingly reduced by 0 02 per cent sodium lauryl sulphate. This indicates that the adsorption of *S senftenberg* to the mucosa might be more dependent on a number of weak bonds such as in hydrophobic interaction whereas the adsorption of *V cholerae* is mediated by other bonds.

In other studies (5 8, 12, 13 30, 34) strains of *S typhimurium* varying with respect to virulence and resistance to phagocytosis were investigated in different *in vivo* models. Since the relative effects of penetration, multiplication and killing were not evaluated, differences in association and invasion for the different strains were hard to assess.

The mechanism by which the bacteria investigated associate with the intestinal mucosa or phagocytic cell is still unrevealed, although a combination of hydrophobic interaction and negative surface charge may favour association and endocytosis. Similar properties may also regulate the recognition of the bacteria by phagocytic cells in the presence of immunoglobulin G complement (31) or secretory immunoglobulin A (9)

The present data thus indicate that bacterial surface characteristics such as charge and hydrophobicity may govern the association or elimination of the microorganisms from the intestinal mucosa. The great association tendency of certain bacteria might, in fact, be an important factor in breaking the intestinal barrier leading to a potentially harmful immune response (24)

We thank Dr *Klas Norrby* Dept. of Pathology for examining the intestinal preparations.

The skilled technical assistance of Mrs. *Britt-Inger Thorin* and Miss *Kerstin H gersten* is gratefully acknowledged.

This work was supported by grant B74-16X 2183-10 from the *Swedish Medical Research Council*

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QUANTITATIVE IMMUNOELECTROPHORETIC STUDIES OF THE L AND S ANTIGENS OF VACCINIA VIRUS

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Gurvin, I., Haukenes, G. & Prante, P. H. Quantitative immunoelectrophoretic studies of the L and S antigens of vaccinia virus. Acta path. microbiol. scand. Sect. B, 85 317–321 1977

The L (10S) and S (7S) antigens of vaccinia virus have been characterized by their sensitivity to heat, to proteolytic enzymes and to periodate. The L antigen was heat labile and inactivated by proteolytic enzymes and periodate. Treatment with trypsin for 1 min indicated that the S antigen was built up of at least two protein subunits of about 3S and 5S. One of the subunits was highly sensitive to heat and to proteolytic enzymes. On further treatment with trypsin the other subunit was split in two components showing partial identity serologically.

Key words: Vaccinia; antigens; quantitative; immunoelectrophoresis.

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Received 7.ii.77 Accepted 29.iv.77

The so-called L-S antigen complex of vaccinia virus has long been known as the major precipitinogens found in vaccinia virus-infected cells (2, 3).

In a previous work (7) we have shown that L and S are distinct antigens which do not form a complex. These antigens can be separated and isolated by sucrose gradient centrifugation having S values of approximately 10 and 7 respectively. The two antigens could also be separated electrophoretically, the L (10S) antigen was more negatively charged. Typical and easily distinguishable patterns were obtained by quantitative crossed, line and crossed line immunoelectrophoresis.

In the present study using the quantitative immunoelectrophoresis method (1, 5) we have examined the effect of proteolytic enzymes, heat and periodate on the L and S antigens.

MATERIALS AND METHODS

Virus

The smallpox vaccine strain from Statens Seruminstitut, Copenhagen, was used in all experiments.

Virus Antigen, Antisera and Separation Methods

Preparation of *vaccinia virus antigen* (VVA), production of *rabbit antisera*, methods for *ultracentrifugation* and *quantitative immunoelectrophoresis* has been described previously (4, 7). In crossed line electrophoresis VVA was applied in ditch between the sample and the antibody-containing agar.

Enzyme Treatment

VVA containing 1–2 mg protein per ml, or L or S fractions isolated by ultracentrifugation, were treated with 0.1 per cent of trypsin (crystalline,

4300 NFμ/mg from Novo Industri A/S Copenhagen) chymotrypsin (crystalline α-chymotrypsin 1160 NFμ/mg Novo) bromelain (Grade II from pineapple 1800 μ/mg, Sigma Chemical Co., St. Louis, Mo., USA) and pronase (ex *Streptomyces griseus* Koch Light Labs., Buckinghamshire England) The action of trypsin was stopped by the addition of an inhibitor (lyophilized from soybean, chromatographically prepared 1 mg inhibits 1.5 mg trypsin) Incubation was performed at 37 C at the time indicated under "Results" Controls were included with buffer added instead of enzyme.

Periodate Treatment

This was performed with 0 017 M potassium periodate for 30 min at room temperature. The reaction was stopped by the addition of glycerol A control with buffer added instead of periodate was included

RESULTS

Treatment with Trypsin

Trypsin appeared to have an almost immediate effect on the S antigen. As will be seen from Fig 1 A and B the S antigen has become split into 2 bands (S-cath and S-an) One band (S-cath) had its peak position very near to the original S peak, and this band had become considerably broadened indicating increased heterogeneity in charge. The other band migrated electrophoretically like the L line, and was accordingly more negatively charged than the original S band. While the bands of the split products both originated from the S line (Fig 1 B) they showed mutual reaction of non identity

The L peak was either unaffected by 1 min's treatment or it became somewhat higher and broadened.

Two or more new bands were regularly found after 1 min's treatment It appears from Fig 1 B that they did not show reaction of identity with the other bands or with the reference lines, and their nature remains obscure

After treatment for 30 min, the L antigen was completely destroyed The S antigen showed 2 split products (S-an 1 and 2) the bands of which fused with spur formation This was also evident when the electrophoresis in the first direction was run for a

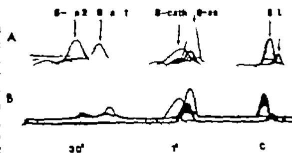


Fig 1 Crossed (A) and crossed line (B) immunoelectrophoresis of VVA (C) and VVA treated with trypsin for 1 and 30 min. ▲ application point.

shorter period (1 h instead of 2 h) (cf Fig. 2 A) Now the more cathodic component (marked S-an 1 and 2, cf below) constituted a shoulder on the other component with spur formation. When line electrophoresis was set up with the 30 min's tryptic digest (Fig 4) one line only could be seen.

When the results of trypsin treatment were recorded at shorter time intervals, it appeared that the cathodic component (S-cath) seen after 1 min's treatment gradually disappeared while the anodic one was split in two, the new one being even more anodic The latter rocket bands were considerably lower in crossed line than in crossed electrophoresis Obviously some of the material was taken up by the reference band, the level of which became slightly heightened.

Heating of VVA at 56 C for 30 min led to total disappearance of the L antigen while the S antigen band became more diffuse and broadened (Fig 2 B) When VVA was treat



Fig 2 A Crossed immunoelectrophoresis of VVA treated with trypsin for 30 min. Electrophoresis in the first direction was run for 1 h (as compared to 2 h in other experiments) B Crossed line immunoelectrophoresis of VVA heated at 56 C for 30 min. C Crossed line immunoelectrophoresis of VVA treated with trypsin for 1 min followed by heating at 56 C for 30 min For control patterns cf. Fig 1 B (C) ▲ application point.

ed first with trypsin for 1 min and then heated, only the anodic one (S-an) of the two split products of the S antigen (cf Fig 1 and 2 C) persisted, which accordingly was heat stable

Treatment with Chymotrypsin

The major pattern obtained from 1 min's treatment with chymotrypsin (Fig 3) was very similar to that obtained with trypsin, but no new precipitation bands appeared. In contrast to trypsin treatment, chymotrypsin did not completely inactivate the L antigen after 30 min. Furthermore, the cathodic component of the 1 min's split product also remained partly intact. The anodic component had two peaks of serological identity (S-an 1 and 2) A third anodic peak (S-an 3) showed spur formation on crossing the S-an 2, indicating partial identity

Serological Comparison of the Trypsin and Chymotrypsin Split Products

Trypsin and chymotrypsin have different substrates for their peptide splitting action. It was therefore of interest to see if comparable split products were serologically identical.

To avoid other interfering lines, the S antigen was first isolated by ultracentrifugation as described earlier (7) Line electrophoresis was performed by placing antigens treated with the enzyme for similar periods adjacently (Fig 4) It is seen that the two cathodic (lower) split products obtained after 1 min's treatment with the two enzymes showed reaction of identity The two anodic (upper) split products differed in density and showed a slight spurring When the products of 1 min's treatment were compared with those of 30 min's, it appeared that two lines showed confluence in the chymotrypsin set-up, which was to be expected since both lines obtained by 1 min's treatment persisted. After trypsin treatment for 30 min, only one line was found, obviously representing the two serologically related split products of the anodic component obtained after treatment

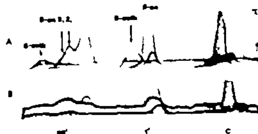


Fig 3 Chymotrypsin treatment of VVA. Crossed (A) and crossed line (B) immunoelectrophoresis before (C) and after 1 and 30 min treatment. ▲ application point.

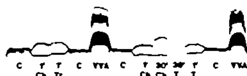


Fig 4 Serological comparison of the trypsin and chymotrypsin split products. Line immunoelectrophoresis of VVA, isolated S antigen (C) and VVA treated with trypsin (T) and chymotrypsin (Ch) for 1 and 30 min

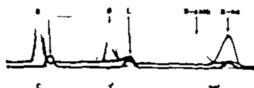


Fig 5 Treatment of VVA with bromelain. Crossed line electrophoresis before (C) and after treatment for 1 and 30 min. ▲ application point.

for 1 min. The cathodic (lower) split products after 1 and 30 min's treatment with chymotrypsin showed reaction of identity while the corresponding anodic (upper) split products showed non-identity

Treatment with Bromelain

The results are shown in Fig 5. The effect of this enzyme was less drastic in that treatment even for 30 min was comparable to that of trypsin for 1 min. The two antigens which after 30 min split off from the S antigen showed reaction of non-identity Both

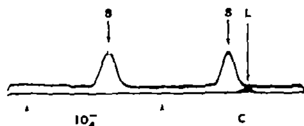


Fig 6 Crossed line immunoelectrophoresis before (C) and after treatment with periodate. ▲ application point.

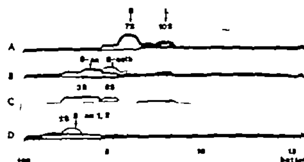


Fig 7 Line immunoelectrophoresis of fractions obtained by ultracentrifugation of VVA (A) and VVA treated with trypsin for 1 min (B and C) and 30 min (C) In A, B and D a reference VVA was placed in a ditch between the applied fractions and the antiserum. Approximate S values are given from a parallel centrifugation of various reference proteins (cf (7))

fused with the original S line. The L antigen was relatively stable to treatment with this enzyme

Treatment with Pronase

The picture obtained by treatment with pronase was very similar to that obtained with bromelain, and no details will be given

Treatment with Periodate

As seen from Fig 6 the L antigen was completely inactivated while the S antigen was unaffected upon treatment with periodate.

Ultracentrifugation of Trypsin Digests

Each of 15 fractions from ultracentrifugation of tryptic digests was examined by line immunoelectrophoresis with and without the reference VVA in a ditch placed between the antibody-containing gel and the fractions.

After treatment with trypsin for 1 min (Fig 7 B and C) the L antigen was still readily distinguished by its S value of about 10 Two additional groups of fractions were isolated with sedimentation coefficients of approximately 3 S and 5 S When the two peak fractions were examined by crossed immunoelectrophoresis, they appeared to represent the two split products of the S antigen (cf Fig 1) the anodic one being the lower molecular one. It was therefore surprising to observe partial reaction of identity between the isolated fractions on line electrophoresis in the presence of reference antigen (Fig 7 B) Without the reference lines (Fig 7 C) the picture was in accordance with that obtained by crossed immunoelectrophoresis.

After 30 min's treatment (Fig 7 D) one band only was found as expected from the findings above (cf Fig. 1 and 4) When examined by crossed electrophoresis, the two bands showing reaction of partial identity were seen.

DISCUSSION

The L antigen which is the 10S and more anodic one of the two was characterized by its heat lability and sensitivity to trypsin and periodate, which led to complete inactivation The antigen was also sensitive to other proteolytic enzymes, but to a lesser degree. We have accordingly not been able to show any heterogeneity of the L antigen The L antigen was sensitive to periodate, which may indicate a sugar as the antigenic determinant group

The S or 7S antigen, however seems to be built up by loosely connected protein subunits and seems to have a region which is highly sensitive to treatment with proteolytic enzymes. The most striking finding was the almost immediate cleaving of the antigen into antigenically different protein subunits of sedimentation coefficient of 3 and 5S and distinct electrophoretic properties (cf Fig 1) Very similar results were obtained by

treatment with 4 proteolytic enzymes. One of the subunits was readily destroyed by further treatment with trypsin and by heating, while the other one gradually split off a more negatively charged group, which was heat stable. The S antigen may thus be regarded to be built up of at least two covalently linked protein subunits. There was no indication of carbohydrate antigenic determinants.

The LS antigen complex has been reported (6) to contain three different precipitation lines. One component was heat stable and split by trypsin into two distinct lines. This pattern conforms well with our findings, but direct comparison of the results is uncertain since different methods were employed.

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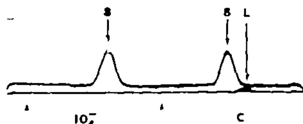


Fig 6 Crossed line immunoelectrophoresis before (C) and after treatment with periodate. ▲ application point.

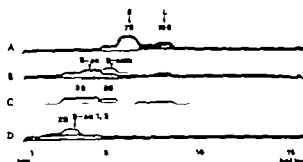


Fig 7 Line immunoelectrophoresis of fractions obtained by ultracentrifugation of VVA (A) and VVA treated with trypsin for 1 min (B and C) and 30 min (C) In A B and D a reference VVA was placed in a ditch between the applied fractions and the antiserum. Approximate S values are given from a parallel centrifugation of various reference proteins (cf. (7))

fused with the original S line. The L antigen was relatively stable to treatment with this enzyme.

Treatment with Pronase

The picture obtained by treatment with pronase was very similar to that obtained with bromelain, and no details will be given.

Treatment with Periodate

As seen from Fig 6 the L antigen was completely inactivated while the S antigen was unaffected upon treatment with periodate.

Ultracentrifugation of Trypsin Digests

Each of 15 fractions from ultracentrifugation of tryptic digests was examined by line immunoelectrophoresis with and without the reference VVA in a ditch placed between the antibody-containing gel and the fractions.

After treatment with trypsin for 1 min (Fig 7 B and C) the L antigen was still readily distinguished by its S value of about 10 Two additional groups of fractions were isolated with sedimentation coefficients of approximately 3 S and 5 S. When the two peak fractions were examined by crossed immunoelectrophoresis, they appeared to represent the two split products of the S antigen (cf Fig 1) the anodic one being the lower molecular one. It was therefore surprising to observe partial reaction of identity between the isolated fractions on line electrophoresis in the presence of reference antigen (Fig 7 B) Without the reference lines (Fig 7 C) the picture was in accordance with that obtained by crossed immunoelectrophoresis.

After 30 min s treatment (Fig 7 D) one band only was found as expected from the findings above (cf Fig 1 and 4) When examined by crossed electrophoresis, the two bands showing reaction of partial identity were seen.

DISCUSSION

The L antigen, which is the 10S and more anodic one of the two, was characterized by its heat lability and sensitivity to trypsin and periodate, which led to complete inactivation The antigen was also sensitive to other proteolytic enzymes, but to a lesser degree. We have accordingly not been able to show any heterogeneity of the L antigen. The L antigen was sensitive to periodate, which may indicate a sugar as the antigenic determinant group

The S or 7S antigen, however seems to be built up by loosely connected protein subunits and seems to have a region which is highly sensitive to treatment with proteolytic enzymes. The most striking finding was the almost immediate cleaving of the antigen into antigenically different protein subunits of sedimentation coefficient of 3 and 5S and distinct electrophoretic properties (cf Fig 1) Very similar results were obtained by

treatment with 4 proteolytic enzymes. One of the subunits was readily destroyed by further treatment with trypsin and by heating, while the other one gradually split off a more negatively charged group, which was heat stable. The S antigen may thus be regarded to be built up of at least two covalently linked protein subunits. There was no indication of carbohydrate antigenic determinants.

The LS antigen complex has been reported (6) to contain three different precipitation lines. One component was heat stable and split by trypsin into two distinct lines. This pattern conforms well with our findings, but direct comparison of the results is uncertain since different methods were employed.

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ENDOCYTOSIS OF *SALMONELLA TYPHIMURIUM* 395 MS AND MR10 BY HELA CELLS

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Kihlström, E. & Nilsson, L. Endocytosis of *Salmonella typhimurium* 395 MS and MR10 by HeLa cells. Acta path. microbiol. scand Sect. B 85 322–328, 1977

Monolayers of HeLa cells were examined for their ability to endocytose *Salmonella typhimurium* 395 MS (wild) and MR10 (chemotype Rd) Monolayers treated with the glycolytic inhibitors iodoacetic acid (IAA) or N-ethylmaleimide (NEM) or the respiratory inhibitor sodium azide (NaN_3) or cytochalasin B (CB) were incubated with *S typhimurium* The numbers of cell associated (intracellular plus cell-membrane attached extracellular) and intracellular bacteria were determined by viable counts, together with the HeLa cell ATP levels. IAA and NEM at concentrations 10^{-4}M and 10^{-3}M decreased significantly the number of intracellular MR10 and the cellular ATP levels, but did not influence significantly the total number of cell associated bacteria except for 10^{-3}M IAA which slightly increased the association. On the other hand, NaN_3 at concentrations 10^{-4}M and 10^{-3}M did not affect the number of associated or intracellular bacteria or the cellular ATP levels CB at concentrations of 5, 10 and 20 µg/ml increased the number of associated bacteria, decreased the number of intracellular bacteria and caused a small decrease in cellular ATP levels. Thus, HeLa cells may internalize *S typhimurium* by an energy-requiring, glycolysis-dependent process. CB had a dose-dependent inhibitory effect on the internalization without influencing significantly the HeLa cell ATP levels This indicates that CB might affect the internalization process by some means other than decreasing the ATP content

Key words Endocytosis HeLa cells *Salmonella typhimurium*

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Received 24 77 Accepted 24 v 77

Microorganisms may enter mammalian cells by at least two different mechanisms phagocytosis and penetration For phagocytosis to occur several conditions must be fulfilled such as appropriate attachment and ingestion. Whereas the attachment is primarily dependent on the properties of the prey the ingestion, an energy-dependent process, is closely linked to the properties of the phagocytic cell Depending on the mode of attachment, there may be activation of the phagocyte reflected as increased oxygen consumption lactic acid production and hexose monophosphate shunt activity This activation is required for internalization of the attached particle (17 22) This process may thus be inhibited by treating the phagocytic cell with

glycolytic inhibitors such as iodoacetate (7) and other agents such as cytochalasin B (25 28)

On the other hand during penetration, the microorganism plays an active role both during attachment and internalization. The metabolic state of the infected cell is of little importance. Penetration has been proposed as the mechanism by which *Toxoplasma gondii* enters HeLa cells (13)

The association of different *Salmonella typhimurium* strains to HeLa cells is dependent on the surface properties of the bacteria. The phagocytosis-sensitive rough (R) strain MR 10 associates to a greater extent than the phagocytosis-resistant smooth (S) strain MS (8, 20) However the association is impeded when the bacteria are exposed to heat or ultraviolet irradiation This indicates that viable *S typhimurium* bacteria influence the attachment and promote internalization. Whether this process is fulfilled through phagocytosis or penetration is not known. The present communication shows that the internalization of *S typhimurium* bacteria can be influenced by metabolic inhibitors in a manner similar to endocytosis of particles by phagocytic cells.

MATERIALS AND METHODS

Bacterial strains The smooth (S) strain *Salmonella typhimurium* 395 MS which is highly virulent for mouse and the rough (R) mutant 395 MR10 (chemotype Rd) derived from it have been described earlier (2 6, 10)

Cultivation of bacteria All strains were kept at 4 C on agar slants before use. The bacteria were inoculated into 15 ml of glucose broth and incubated at 37 C for 18 h. The bacteria were harvested by centrifugation (1100 × g, 15 min) washed twice in phosphate buffered saline solution (PBS) pH 7.3, and suspended to 4.0×10^7 bacteria/ml (estimated by Turner spectrophotometer at 650 nm) in Earle balanced salt solution (EBSS) pH 7.3 (Flow Laboratories, Irvine, Scotland)

Mammalian cell culture The HeLa cell line (ATCC strain CCL 2, human serum research grade) was obtained from Flow Laboratories. Specimens of cells were kept in liquid nitrogen and initiated at intervals not longer than three months The cells were grown in Eagle' minimal essential medium supplemented with 10 per cent foetal bovine serum, 100 IU of penicillin and 100 IU of streptomycin/ml. Cells were maintained as monolayers in glass bottles in a humidified incubator with an atmosphere of 5 per cent CO and 95 per cent air at 37 C. The monolayers were treated with 0.25 per cent trypsin to detach the cells, and the detached cells were used to prepare new monolayers in tissue culture petri dishes, 50 × 15 mm. The petri dishes were seeded with 2 ml HeLa cell suspension and 1 ml fresh medium. The dishes were incubated for approximately 72 h in the humidified incubator with changes of medium every 24 h, the last change being without penicillin and streptomycin. The cells were not contaminated with mycoplasma when tested regularly by staining with orcein (5) All cell culture material was purchased from Flow Laboratories, Irvine, Scotland.

Interaction procedure After incubation for 72 h, the medium was poured off from the petri dishes, the bottoms were rinsed three times in 37 C PBS, pH 7.3 3 ml of bacterial suspension was added and the petri dishes were incubated again in the humidified incubator After incubation for 3 h the monolayers were rinsed three times in 37 C PBS, pH 7.3 and detached from the petri dishes with 0.25 per cent trypsin and rubber "policeman The HeLa cells were suspended in 3 ml of EBSS plus 24 ml of PBS and disintegrated with modified LoX press at an extrusion pressure of 3.3×10^8 to 6.1×10^8 Pa (8, 23) Samples were removed from the homogenate for viable counts. Where indicated the monolayers were incubated for 1 h in different concentrations of iodoacetic acid (IAA) (Kebo AB, Stockholm, Sweden) sodium azide (NaN) (Fluka AG Buchs SG Switzerland) and N-ethylmaleimide (NEM) (BDH Chemicals Ltd, Poole England) or for 30 min in cytochalasin B (CB) (Ega-Chemie KG Steinheim, West Germany) and washed three times in 37 C PBS, pH 7.3 before the bacteria were added CB was present during the 3-hour incubation with the bacterial suspension, but this was not the case for IAA, NaN and NEM, since they were shown to have an irreversible effect on HeLa cell ATP levels and decreased the bacterial viability I order to determine the intracellular bacterial fraction, the bacterial suspension was poured off from the petri dishes, the monolayers were washed three times in 37 C PBS, pH 7.3 and 40 µg gentamicin/ml was added for 20 min before disintegration. Viable counts were then performed (9)

Cellular ATP determinations Total HeLa cell ATP levels were determined by luciferin-luciferase assay in which the production of light is related to the amount of ATP present. The analytical equipment, reagents and luciferase assay have been described earlier (12) HeLa cell mono-

layers were incubated for 3 h in EBSS or in 5 10 or 20 µg/ml of CB, or preincubated for 1 h in 10^{-4}M or 10^{-3}M IAA, NEM or NaN_3, rinsed three times in 37 C PBS pH 7.3 and then incubated for 3 h, so as to mimic the conditions during incubation with bacteria. The monolayers were washed three times in PBS pH 7 3 and detached from the petri dishes. The ATP was extracted by boiling for 5 min in Tris-EDTA buffer pH 7 75 and assayed for ATP In the ATP assay system, corrections were made for the influence of other biological components and metabolic inhibitors. Cell protein was measured according to *Lowry et al.* (11) with human serum albumin (Kabi, Stockholm Sweden) as standard

RESULTS

Effects of IAA NEM NaN_3 and CB on the HeLa cell membrane permeability To determine the effects of the metabolic inhibitors on HeLa cells, two approches were used

A Vital staining HeLa cell monolayers were treated with 10^{-3}M IAA NEM NaN_3 or with 20 µg/ml CB for 3 h and then stained with Trypan blue (0 05 per cent w/v in normal saline) The metabolic inhibitors did not affect the number of HeLa cells that excluded Trypan blue.

B Effect on intracellular bacterial survival. HeLa cell monolayers were incubated for 3 h with 3 ml 4 × 10^7 MR10/ml, the bacterial suspension was poured off and the monolayers were washed in PBS The monolayers were then treated with different concentrations of IAA, NEM or NaN_3 for 1 h, or with 20 µg/ml of CB for 30 min, washed in PBS treated with 40 µg/ml of gentamicin for 20 min washed in PBS and disintegrated, and viable counts were performed on the homogenates. The result of this treatment with metabolic inhibitors *after* initial interaction between bacteria and HeLa cells is shown in Fig 1 IAA and NaN at a concentration of 10⁻ M 10^{-4}M NEM and 20 µg/ml of CB did not influence significantly the intracellular bacterial survival, whereas 10^{-3}M NEM decreased the number of intracellular bacteria to 5 per cent of the value with EBSS alone.

Effects of IAA NEM NaN and CB on bacterial growth The changes in the viable population of *S typhimurium* during the experimental conditions with metabolic inhibitors were determined by adding the number of viable bacteria in the medium, including the three rinsings, to that of the disintegrated HeLa cells. IAA, NEM NaN_3 and CB did not change either the MS or the MR10 population by more than 20 per cent of the value when the bacteria were incubated with HeLa cells in EBSS without inhibitors (Table 1 2)

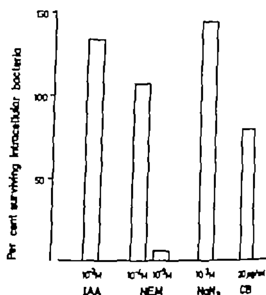


Fig 1 Effects of IAA, NEM, NaN_3 and CB on the number of surviving *S typhimurium* 395 MR 10 after initial interaction between bacteria and HeLa cells for 3 h The results are expressed as percentage of the number of intracellular bacteria in HeLa cells incubated in EBSS without inhibitors

TABLE I *Amount*) *of S* typhimurium *395 MR 10 in the HeLa Cell System after Incubation with Different Metabolic Inhibitors* b)

	10^{-4}M	10^{-3}M
IAA	*119 per cent*	95 per cent
NEM	99 per cent	84 per cent
NaN	115 per cent	99 per cent

a) Sum of the bacteria in the extracellular fluid the three rinsings and the homogenate
b) The HeLa cells were preincubated for 1 h in IAA, NEM or NaN_3, washed in PBS and then incubated for another 3 h with a bacterial suspension in EBSS before viable counts were performed. The results are expressed as percentage of the number of bacteria in the HeLa cell system incubated in EBSS without inhibitors.

TABLE 2. *Amount* [a] *of S.* typhimurium *395 MS and MR10 in the HeLa Cell System after Incubation with Cytochalasin B* [b])

	5 µg/ml	10 µg/ml	20 µg/ml
MS	90 per cent	94 per cent	
MR10	92 per cent	106 per cent	81 per cent

a) Sum of the bacteria in the extracellular fluid, the three rinsings and the homogenate.
b) The HeLa cells were preincubated for 30 min in CB, washed in PBS and then incubated for another 3 h with bacterial suspension in CB before viable counts were performed. The results are expressed as percentage of the number of bacteria in the HeLa cell system incubated in EBSS without inhibitor.

Effects of IAA, NEM, NaN₃ and CB on the association of S. typhimurium *bacteria with HeLa cells.* IAA at a concentration of 10^{-3}M slightly decreased, while 10^{-4}M IAA increased the association of MR10 approximately 1.5-fold ($P < 0.05$, Student's t-test). NEM at a concentration of 10^{-3}M reduced the association by approximately 50 per cent, but 10^{-4} had minimal effect. NaN_3 at concentrations 10^{-4}M and 10^{-3}M did not influence the association at all. CB caused an increase in the association of both MS and MR10. The greatest effect was achieved with the highest concentration used: 10 µg/ml for MS caused a 2-fold increase and 20 µg/ml for MR10 caused a 4.5-fold increase ($P < 0.02$, Student's t-test, Fig. 2). Dimethyl sulphoxide (DMSO) (0.5 per cent) increased the association of MR10 approximately 1.5-fold.

Effects of IAA, NEM, NaN₃ and CB on the number of internalized S. typhimurium *bacteria in HeLa cells.* IAA at a concentration of 10^{-4}M caused an approximately 2-fold ($P < 0.05$, Student's t-test) and 10^{-3}M a 23-fold ($P < 0.01$, Student's t-test) decrease in the number of intracellular MR10. NEM at a concentration of 10^{-4}M caused a 60-fold inhibition of the internalization of MR10 ($P < 0.001$, Student's t-test) and 10^{-3}M a 170-fold inhibition ($P < 0.001$, Student's t-test). On the other hand, NaN_3 at concentrations 10^{-4}M and 10^{-3}M did not influence the internaliza-

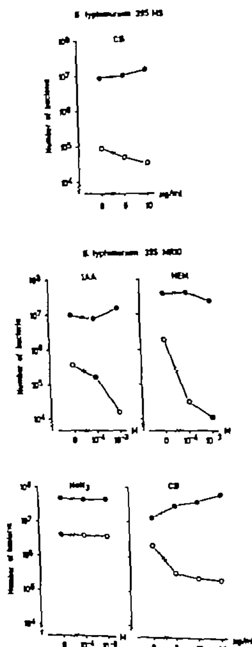


Fig. 2. Total number of HeLa cell associated (●—●) and intracellular (○—○) *S. typhimurium* 395 MS in the presence of CB (A) and *S. typhimurium* 395 MR10 after pretreatment with IAA, NEM or NaN_3 or in the presence of CB (B). Logarithmic scales on abscissa and ordinate.

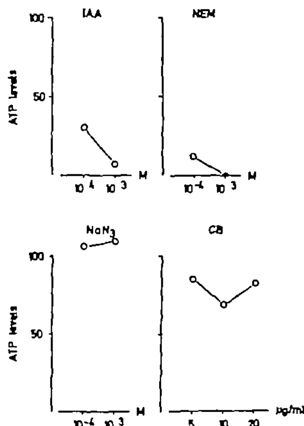


Fig 3 Effect of IAA NEM NaN_3 and CB on the HeLa cell ATP levels. The results are expressed as percentage of ATP levels in HeLa cells treated with EBSS alone and related to the protein content of the HeLa cells. Logarithmic scale on abscissa.

tion of MR10 Continuous incubation with 5 µg CB/ml caused an approximately 6-fold inhibition of the internalization of MR10 By increasing the concentration to 10 µg/ml and 20 µg/ml the inhibition increased 8.5-fold ($P<0.05$ Student's t test) and approximately 10-fold ($P<0.10$ Student's t test) respectively The effect of CB on the internalization of MS was also dose-dependent 5 µg/ml caused a 2 fold ($P<0.05$ Student's t test) and 10 µg/ml a 3 fold ($P<0.01$ Student's t test) decrease in the internalization (Fig 2) DMSO (0.5 per cent) did not influence significantly the number of internalized bacteria.

Effects of IAA NEM NaN and CB on the ATP content of HeLa cells The ATP level in HeLa cells incubated with EBSS was approximately 6.5×10^{-14} mol/cell. The glycolytic inhibitors IAA and NEM decreased the cellular ATP content to 0 per cent to 30 per cent of the value in non-treated cells (Fig. 3) NaN_3 had only small effect on the ATP content, while 5 to 20 µg/ml of CB decreased the ATP levels to 60 per cent to 85 per cent of the control value (Fig 3)

DISCUSSION

The internalization of *Salmonella typhimurium* into HeLa cells was inhibited by the glycolytic inhibitors iodoacetic acid and N-ethylmaleimide and by the mold metabolite cytochalasin B but not by the respiratory inhibitor sodium azide. In parallel experiments, there was a decrease in the cellular ATP levels. These results indicate that the uptake of *Salmonella typhimurium* bacteria proceeds by an energy-dependent process similar to phagocytosis.

HeLa cells may generate ATP both via the glycolytic and respiratory pathways (16) The sulphydryl (-SH) group inhibitors IAA and NEM (24) had a pronounced inhibitory effect on the internalization of *MR10* bacteria into HeLa cells. The effect on the total number of cell associated bacteria was small and showed that the inhibitory effects of IAA and NEM were against the internalization process. NaN_3 had no effect either on the number of internalized MR10 or on cellular ATP content. Whereas it is known that respiratory inhibitors do not influence significantly the phagocytic process in polymorphonuclear leukocytes (17) the pinocytosis of *HRP* by fibroblasts was decreased by 10^{-3}M NaN_3 (19) but not the ATP levels. *Vlodavsky et al* (27) found a decrease in the ATP content in transformed fibroblasts treated with 5×10^{-3}M NaN However this inhibition did not occur when the cells were grown in 1 mM glucose and 5×10^{-3}M NaN_3. The HeLa cells used in this study were grown in 5.6 mM glucose which may explain the inability of NaN_3 to inhibit the cellular ATP content. The ATP generated from glycolysis of endogenous and exogenous carbohydrates may be sufficient to internalize MR10 bacteria into HeLa cells.

CB affected both the total number of cell-

associated bacteria and the number of internalized MS and MR10 bacteria. These effects were dose-dependent increased concentration of CB increased the total number of cell-associated bacteria and decreased the number of internalized bacteria. Endocytosis is accompanied by movement of host cell membrane around its prey. Ruffling of the membrane may arise by contraction of bundles of microfilament located just under the plasma membrane (4). The inhibition of endocytosis and cell movement by CB might be ascribed to its effect on microfilament. *Schroeder* (18) has demonstrated decomposition of the microfilaments in CB-treated HeLa cells. CB also disaggregated actin in extracts from HeLa cells (26). However CB did not change the actin content of HeLa cell membranes significantly (5). Thus, CB may alter functionally the structure of the microfilaments. If CB affected the internalization of bacteria into HeLa cells by interfering with the function of microfilaments, one would expect this to occur also in cells with normal ATP levels. However CB decreased the ATP content, although not as efficiently as IAA and NEM did. In most cases these glycolytic inhibitors also decreased the number of intracellular bacteria to a greater extent than CB. IAA at a concentration of 10^{-4}M depressed the cellular ATP content to 30 per cent and inhibited the internalization of the bacteria to approximately 40 per cent. The corresponding values for 20 μg/ml of CB were 80 per cent and 10 per cent, respectively. Five, 10 and 20 μg/ml of CB decreased the number of intracellular bacteria more than 10^{-4}M IAA, despite higher ATP levels. This indicates that CB inhibited the internalization by mechanisms other than IAA, e.g. by interfering with the function of the microfilaments.

CB increased the total number of HeLa cell-associated bacteria, especially MR10. CB-treated Ehrlich ascites cells have a lower mean negative electrophoretic mobility than non-treated cells (15). *Mayhew et al.* (15) suggested that CB alters the expression of ionized sialic acid groups at the electrokinetic surface. The reduced negative charge density might reduce the electrostatic repulsive forces between host cells and microorganisms. This should be more pronounced for MR10 bacteria, which have a net negative surface charge, than for MS bacteria, which are virtually uncharged (14, 21).

Our results show that HeLa cells have the ability to internalize viable *S. typhimurium* by a process similar to phagocytosis. Earlier experiments have demonstrated a decreased tendency for heat inactivated and UV-inactivated *S. typhimurium* to associate with HeLa cells (8). It has been reported that heat killed Pseudomonas are less liable to phagocytosis and hexose monophosphate stimulation than viable bacteria (1). However this may vary for different bacterial species. In non-professional phagocytes like HeLa cells, mere attachment may not be sufficient to produce phagocytosis. Heat-sensitive factors (soluble or membrane-bound) are required to activate the HeLa cell in a way that leads to phagocytosis. These questions are being investigated at present.

The skilled technical assistance of *Kerstin Hagerstén* is gratefully acknowledged. This work was supported by grant B-76-16X-2183-10 from the *Swedish Medical Research Council* and by the *Swedish Board of Technical Development*.

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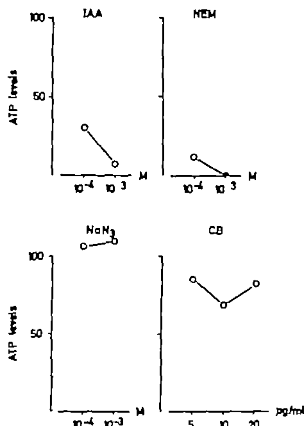


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Acta path. microbiol. scand. Sect. B, 85 329–333, 1977

INFECTION OF *CLETHRIONOMYS G GLAREOLUS SCHREB* (RED MICE) WITH *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS* INJECTED SUBCUTANEOUSLY

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Jespersen, A. Infection of *Clethrionomys g glareolus S hreb* (red mice) with *Mycobacterium tuberculosis* and *Mycobacterium bovis* injected subcutaneously Acta path. microbiol. scand. Sect B, 85 329–333 1977

Groups of red mice were injected with doses from 10 mg to 10^{-4} mg semidried culture of strain of *M tuberculosis* and with doses from 10- to 10^{-4} mg of a strain of *M bovis*. Some animals were killed about 1½ and 3 months after injection and the remainder lived until death occurred spontaneously The number of tubercle bacilli in the organs was evaluated by microscopy of smears, in some cases by quantitative culture. Among the mice injected with *M t berculosis* in doses of up to about 2 million iable units, not one case of death occurred which could be attributed to tuberculosis. The utopsy findings consisted exclusively of lesions at the site of injection and in the regional lymph glands. Quantitative culture showed growth of few iable units in the lymph glands, spleen or lungs, but no sign of progressi infection. Out of 10 mice injected with giant dose of 3×10^{7} iable units, only tw died of tuberculosis. *M bovis* provoked fatal tuberculosis in all animals injected with doses from 6.9 million to 7 viable units. Severe caseous lesions developed at the site of injection, in the lymph glands, in the lungs, and often also in liver and spleen. The number of bacteria in the organs was enormous, particularly in the spontaneously dead animals. The survival times, which were dependent on dosage, aried from 51 to 159 days.

Key words *Mycobacterium t berculosis Mycobacterium bovis* subcutaneous infection species differentiation *Clethrionomys g glareolus S hreb* (red mice)

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Received 2 77 Accepted 2. 1.77

Pilot studies have shown that red mice, in the same way as rabbits, are susceptible to *M bovis* injected intraperitoneally but are resistant to *M tuberculosis* A short survey of these experiments was given in a publication from 1954 (*Jespersen)* The present and three subsequent papers comprise studies of the course of the infection in red mice injected subcutaneously intraperitoneally or intra venously with varying doses of strains of the two species.

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paper and weighed, then physiological saline was added so that the suspension contained 1 mg culture in 0 2 ml. From this suspension *dilutions* in ratio 1:9 were prepared with saline to 10^{-6} mg per 0 2 ml. Inoculation of 0 1 ml of suitable dilutions on to each of 10 tubes of Löwenstein-Jensen medium showed that 1 mg culture contained 17 × 10^{6} viable units of V 11381 H, 69 × 10^{6} viable units of T 3474 B (Expt. 1) and 5 × 10^{6} viable units of E 10683 H (Expt. 2)

Infection The mice were injected subcutaneously with 0 2 ml of the various dilutions in the right inguen.

Registration The degree of tuberculosis and the number of bacteria in the organs of both the killed and spontaneously dead animals were recorded by means of an index based on macroscopical and microscopical findings and results of culture.

Tuberculous Index

0 Organs normal, culture negative.
1 No definite specific lesions, culture positive.
2 Small tuberculous lesions, mainly of regressive nature.
3 Slight progressive tuberculosis.
4 Medium progressive tuberculosis.
5 Severe progressive tuberculosis.

Bacterial Index

0 No bacteria.
1 Microscopy negative, culture positive.
2 1 20 bacteria by microscopy (Ziehl-Neelsen stained smears)
3 > 20 bacteria to 1–2 bacteria per sight field.
4 1–2 to 10–20 bacteria per sight field.
5 Innumerable bacteria per sight field.

RESULTS

Experiment 1

The survival times, tuberculous index and bacterial index for the killed and spontaneously dead animals injected with varying doses of the two species are shown in Table 1

M tuberculosis In none of the groups were there any deaths caused by *M tuberculosis* as can be seen from the results of culture Neither were the survival times of the spontaneously dead animals in the individual groups affected by the number of

Red Mice Injected Subcutaneously with Varying Doses (mg, Viable Units) of a Strain of M. tuberculosis *and* M bovis

Surv. time	Tub index	Bact. index	Surv time	Tub. index	Bact. index	Surv. time	Tub. index	Bact. index
dose and number of viable units								
10− 2			10^{-4} 0.2					
k 54	0	0	k 54	0	0			
k 54	0	0	k 54	0	0			
k 97	0	0	k 96	0	0			
k 97	0	0	k 96	0	0			
+304	1	0	+356	0	0			
+307	0	0	+378	0	0			
dose and number of viable units								
10^{-4} 69			10^{-4} 7			10^{-4} 0 7		
k 50	4	1 7	+ 1	0		k 52	0	0
k 50	3	0.9	k 53	4	1.3	k 52	0	0
k 94	4	4.0	+ 85	5	5 0	k 94	0	0
k 94	4	1 3	k 94	4	2 8	k 94	0	0
105	3	4.5	+115	5	3.3	+120	5	4 4
159	5	4 1	+156	5	4.3	+240	3	3 1

MATERIAL AND METHODS

Experimental

Experiment 1 Groups of mice, with six animals in each, were injected subcutaneously with doses varying from 10^{-1} to 10^{-8} mg semidried culture of a strain of *M tuberculosis* (V 11381 H) or a strain of *M bovis* (T 3474 B) The bacterial suspensions were the same as those used for examination of the intraperitoneal infection and both experiments were carried out on the same day (*Jespersen* 1977)

Two mice from each group were killed 1½ and 3 months after the injection and the remainder lived until death occurred spontaneously Smears were made from the abscess at site of injection, from the lymph glands, liver spleen and lungs, and were stained by the Ziehl Neelsen method In cases where no lesions were observed macroscopically or where the lesions were small or uncharacteristic, quantitative culture was made from lymph glands, spleen and lungs. Before inoculation on to four Löwenstein-Jensen tubes, the organ suspensions were treated with 4 per cent natron

Experiment 2 Since 10^{-1} mg *M tuberculosis* had only a weak effect, examinations were made to ascertain whether a dose of 10 mg was able to provoke progressive infection A group of 10 mice was injected with 10 mg semidried culture of another virulent strain of *M tuberculosis* (E 10883 H) and the animals were allowed to live until they died spontaneously On autopsy culture was made from lymph glands, liver spleen and lungs.

Experimental animals The animals were bred at the farm belonging to Statens Seruminstitut, and were 2–5 months old at the start of the experiments. They were distributed at random into earthenware jars with one mouse in each Each experimental group consisted of equal numbers of males and females

Bacterial strains The strain of *M tuberculosis* V 11381 H and the strain of *M bovis* T 3474 B were chosen fortuitously and were freshly isolated. The strain of *M tuberculosis* E 10883 H was an approximately 3-year-old laboratory strain, the virulence of which had been maintained by inoculation on to Löwenstein-Jensen medium alternating with animal passages. Virulence determination showed that all three strains were highly virulent for guinea pigs. The strain of *M bovis* was highly virulent and the strains of *M tuberculosis* weakly virulent for rabbits. There was no difference in the virulence of the *M tuberculosis* strains.

Bacterial suspension for infection The strains were cultured in Besredka fluid medium and the culture was used when about 10 days old. The sediment was collected by pipette, dried on filter

TABLE 1 *Survival Times (Days) Tuberculosis Index and Bacterial Index for Killed and Spontaneously De… a Strai…*

Surv. time	Tub. index	Bact. index	Surv time	Tub index	Bact. index	Surv. time	Tub index	Bact. index
						M tuberculosis strain V 11381 …		
10^{-1}	17×10^{5}		10^{-3}	17×10^{3}		10^{-5}	17 × 10 …	
k 54	1	0 8	k 54	2	1.3	k 54	2	0 8
k 54	2	1.5	k 54	1	0.3	k 54	0	0
k 96	1	0.3	+ 86	0	0	k 97	0	0
k 96	2	0 8	k 97	0	0	k 97	0	0
+ 178	1	0.5	+ 166	1	0.3	+ 325	0	0
+ 349	0	0	+ 399	0	0	+ 450	1	0 3
						M bovis strain T 3474 …		
10–	69×10^{6}		10^{-3}	69×10^{4}		10^{-5}	69 × 10 …	
k 50	5	4 0	k 50	4	3 0	+ 32	5	2.9
k 50	5	4 1	+ 53	4	1.9	k 53	5	0 9
+ 51	5	4 0	+ 54	5	4.2	+ 74	5	5 0
+ 54	5	5 0	+ 67	5	5 0	+ 82	5	5.0
+ 58	5	2.3	+ 82	5	5 0	+ 104	5	4 2
+ 86	5	3.5	+ 116	5	4.3	+ 147	5	4 4

k = killed + = spontaneously dead.

paper and weighed, then physiological saline was added so that the suspension contained 1 mg culture in 0.2 ml. From this suspension dilutions in ratio 1:9 were prepared with saline to 10^{-8} mg per 0.2 ml. Inoculation of 0.1 ml of suitable dilutions on to each of 10 tubes of Löwenstein-Jensen medium showed that 1 mg culture contained 17 × 10^{6} viable units of V 11381 H, 69 × 10^{6} viable units of T 3474 B (Expt. 1) and 5 × 10^{6} viable units of E 10083 H (Expt. 2)

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Surv time	Tub. index	Bact index	Surv time	Tub. index	Bact. index	Surv time	Tub. index	Bact. index
dose and number of viable units								
10^{-7} 2			10^{-8} 0.2					
k 54	0	0	k 54	0	0			
k 54	0	0	k 54	0	0			
k 97	0	0	k 96	0	0			
k 97	0	0	k 96	0	0			
+304	1	0	+356	0	0			
+307	0	0	+378	0	0			
dose and number of viable units								
10^{-6} 69			10^{-7} 7			10^{-8} 0 7		
k 50	4	1	+ 1	0		k 52	0	0
k 50	3	0 9	k 53	4	1.3	k 52	0	0
k 94	4	4.0	+ 85	5	5.0	k 84	0	0
k 94	4	1 3	k 94	4	2 8	k 94	0	0
+103	5	4.5	+115	5	3.3	+120	5	4 4
159	5	4 1	+156	5	4.3	+240	5	3 1

bacteria injected. The autopsy findings consisted exclusively of local lesions. During the first period (up to day 54) some of the animals in several of the groups had developed *a small abscess at the site of* injection. In others there were remains of an abscess containing a few tubercle bacilli. Of the regional lymph glands, the lumbar glands were somewhat enlarged During the second period (day 54 to day 97) and the third period (after day 97) there were no longer signs *of abscess and the lumbar glands* were enlarged only in two mice in the 10^{-1} group killed on day 96 During the whole experimental period quantitative culture gave growth of a few viable units in lymph glands, spleen or lungs in the 10^{-1} 10^{-4} and 10^{-5} groups. The number of bacteria decreased with time *particularly in spleen and lungs* In the lymph glands the bacteria persisted for a long time

M bovis Doses from 69×10^{5} to 7 viable units provoked progressive tuberculosis in all animals. The survival times became prolonged the smaller the dose, except for the small doses, where *no differences could be seen* In the 10^{-8} group (0 7 viable units) two mice died of generalized tuberculosis after 120 and 240 days the other mice in the group showed no signs of infection

Period up to day 53 All animals showed a pea-sized to nut kernel sized abscess at the site of injection In the 10^{1} group this had often become evacuated by perforation of the skin. The regional lymph glands (subcutaneous gland in the inguen and the lumbar gland) were caseous and very enlarged particularly *the last named which* were up to the size of datekernels. The lungs of animals in the 10^{-1} and 10^{-} groups contained numerous wholly or partially caseous tubercles, while there were a few gray semi transparent tubercles in animals *in the 10^{-3} to 10^{-7} groups*. The spleen was enlarged—10–20 times normal volume—in the 10^{-} and 10^{-} groups, and was 2–3 times normal volume in the other groups Both spleen and liver occasionally contained tubercles. *The number* of bacteria was large in the 10^{-1} group in the 10^{-} to 10^{-7} groups there were numerous bacteria in the abscess and the lumbar glands, but still few in liver spleen and lungs.

Period from day 54 to day 94 All the mice —also those injected with small doses—had severe or medium progressive tuberculosis, *and the differences in the development of the* infection in the various groups were now almost equalized The lesions in the organs were generally larger than during the first period. The lymph glands in the abdomen and thorax were almost or completely caseous. The spleen was very enlarged (10 to 30 times normal volume) and always contained tubercles. Half of the mice had tubercles in the liver There were large caseous tubercles in the lungs, and in some cases one or more lobes were completely caseous. The number *of bacteria in the lymph glands, spleen* and lungs was generally enormous.

Period after day 94 Autopsy and bacterial findings resembled generally the findings in the spontaneously dead animals in the previous experimental period. The abscess at the site of injection had a tendency to perforate *also in mice injected* with a small dose. The mouse that survived the longest (died on day 240) had large, fluid filled abscesses *in the* spleen.

Experiment 2

The survival times, tuberculosis index and bacterial index for the spontaneously dead animals injected with 10 mg culture (3×10^{7} viable units) of *M tuberculosis* strain *E 10883 H are shown in Table 2*

Two mice died on days 191 and 197 of tuberculosis. The autopsy findings in both animals were almost identical The lungs were dark red and solid and the surface was covered with irregularly outlined, *confluent* gray tubercles. There were no tubercles in liver and spleen but the spleen was moderately enlarged The lymph glands were slightly enlarged but not caseous. There were some tubercle *bacilli in the glands, liver and spleen,* and very many in the lungs.

In the two mice that died on day 177 and day 224 the cause of death was not tuber

TABLE 2. *Survival Times (Days), Tuberculous Index and Bacterial Index for Red Mice Injected Subcutaneously with 10 mg (3 × 10⁷ Viable Units) of a Strain of M. tuberculosis (E 10883 H)*

Survival time	130	154	177	181	187	191	197	224	263	371
Tuberculosis index	1	1	1	1	1	3	3	1	1	1
Bacterial index	0.5	0.8	2.0	1.0	0.3	3.5	3.1	1.8	0.5	0*

* Culture not performed

culosis. Macroscopically there were no signs of tuberculosis. However, the bacterial index was relatively high, and therefore it cannot be excluded that the infection was in a progressive phase. The remaining mice were normal macroscopically. Tubercle bacilli were found constantly in the lymph glands, but there were very few or none at all in liver, spleen and lungs.

DISCUSSION

Red mice are highly resistant to *M. tuberculosis* injected subcutaneously. The smallest dose that could provoke a progressive infection was about 30 million viable units—a giant dose for an animal weighing 15–20 g. Furthermore, the infection only became progressive in four out of 10 animals and only two of them died (after 6 to 7 months). However, it must be emphasized that the animals in these experiments, and particularly in experiment 2, were not as capable of surviving as those in other experiments. The results of culture indicate that the tubercle bacilli persist throughout the lifetime somewhere or other in the organism, mainly in the lymph glands. This applies also to animals injected with a small dose. Had the animals lived longer, it is therfore probable that more cases of death due to tuberculosis would have occurred. It has been shown (*Jespersen* 1974) that in another member of the vole family, i.e. *Arvicola terrestris* (vole rat) a few viable units of a strain of *M. tuberculosis* injected intravenously can remain viable for up to 3 years.

The red mouse is exceedingly susceptible to *M. bovis*. A few viable units, or probably even a single bacterium, were sufficient to provoke a fatal infection. It is characteristic for the *M. bovis* infection that the bacteria multiply excessively and that caseous lesions develop everywhere in the organism. During transport of tubercle bacilli from the subcutis via the lymph vessels to the blood, some of the bacteria are retained in the lymph glands, and therefore lesions at the site of injection and in the regional lymph glands dominate.

In consequence of the high resistance of red mice to *M. tuberculosis* injected subcutaneously, it would be desirable to use that route for differentiation between *M. tuberculosis* and *M. bovis*. However, since the abscess at the site of injection often perforates the skin and evacuates large amounts of contagious material, it is preferable to use the intraperitoneal route, which is the easiest, or the intravenous route, which is the most reliable.

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Acta path. microbiol. scand. Sect. B, 85 334–340 1977

SURFACE-CHARGE CHARACTERISTICS OF SMOOTH AND ROUGH *SALMONELLA TYPHIMURIUM* BACTERIA DETERMINED BY AQUEOUS TWO PHASE PARTITIONING AND FREE ZONE ELECTROPHORESIS

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Stendahl, O., Edebo L., Magnusson, K. E., Tagesson, C. & Hjertén, S Surface-charge characteristics of smooth and rough *Salmonella typhimurium* bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. Acta path. microbiol. scand. Sect. B, 85 334–340 1977

Aqueous biphasic partitioning of *Salmonella typhimurium* S and R bacteria in a system containing 6 2 per cent (w/w) dextran 500 and 4 4 per cent (w/w) poly(ethyleneglycol)6000 (PEG) was similar to the partition of the corresponding surface lipopolysaccharide (LPS) Further partition analysis with charged PEG showed that S bacteria and their LPS exposed very little charge, whereas R bacteria and their LPS showed a conspicuous negative charge at neutral pH Free zone electrophoresis also indicated that the S bacteria have a much lower surface charge density than the R bacteria and accordingly a different surface structure. Thus, the physico-chemical properties of the bacterial surface seem to be determined to a great extent by the characteristics of the cell surface LPS

Key words *Salmonella typhimurium* lipopolysaccharide surface charge aqueous two-phase systems free zone electrophoresis.

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Received 27 v 77 Accepted 27 v 77

Physico-chemical surface properties of smooth (S) *Salmonella typhimurium* 395 MS and a series of R mutants derived from it have been related to the chemical composition of the cell wall lipopolysaccharide (LPS) (7) virulence (4) and phagocytosis (16 17)

It has been shown recently by aqueous two-phase partitioning with hydrophobic PEG-palmitate that MR10-bacteria (Rd mutant) and MR10-LPS are liable to hydrophobic interaction whereas MS (wild type) bacteria and MS-LPS are not. The tendency to ionic interaction was also reduced in the S strain

(15) Since both hydrophobicity and charge are important for the outcome of the contact between the microorganism and the host cell (17 19) experiments were undertaken firstly to determine the surface-charge characteristics of different *Salmonella typhimurium* bacteria and secondly to assess to what extent these properties could be attributed to the surface LPS.

Partition in a dextran-PEG two-phase system containing positively (trimethylammo-) or negatively (sulphonate-) charged PEG and free zone electrophoresis discerned a substantially greater negative surface charge on MR10 than on MS bacteria and LPS.

MATERIALS AND METHODS

Bacteria The smooth, mouse virulent *Salmonella typhimurium* 395 MS and LT2, and the mutants derived from them have been described earlier (11) All strains were kept at 4 C on agar slants before use. Bacteria were grown, harvested and heat killed as described previously (15) The uridine diphosphate (UDP)-galactose(gal)-4-epimerase-less mutant, LT2 M1 was cultivated according to *Lindberg et al* (10) in glucose medium in the absence of galactose yielding LT2 M1 (glu) or in the presence of galactose giving LT2 M1 (gal)

LPS preparation LPS was extracted from S bacteria (MS, LT2 M1 grown with galactose) by the phenol/water method described by *Westphal et al* (21) and from R bacteria (MR10 LT2 M1 grown without galactose) by the phenol/chloroform/petroleum ether method according to *Galanos et al.* (5)

Labelling of bacteria and LPS The bacteria were labelled with ^{51}Cr as described previously (15) The LPS was labelled with ^{51}Cr in the following way (2) LPS was suspended in distilled water to a concentration of 5 mg/ml. 7 ml of this suspension was mixed with 0 5 ml $Na_2{}^{51}CrO$ (*0 5* mCi) and incubated at 37° C After 24 h the mixture was dialysed against distilled water until no significant activity could be detected in the dialysate Finally the labelled LPS was concentrated to approximately 10 mg/ml.

Phase systems A two-phase system (1) was prepared from stock solutions of 20 per cent PEG 6000 (Carbowax 6000 Union Carbide, New York, N Y) 20 per cent dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden) 0.1 M tris-(hydroxymethyl)aminomethane (tris) buffer pH 7.0, and distilled water and was allowed to equilibrate at 4 C overnight. The total system contained 4 4 per cent (w/w) PEG and 6.2 per cent (w/w) dextran in 0.05 M tris buffer (pH = 7.2) In some experiments the influence on partition by charged PEG was tested by substituting part of the PEG with charged PEG (8) Positively charged trimethylamino($(CH_3)_3N^+$–)-PEG called TMA–PEG and negatively charged sulphonate-(SO^- –)-PEG, called S–PEG were kindly supplied by Dr *G Johansson* Umeå, Sweden.

Partition analysis Single step Two ml of bottom phase and two ml of top phase were pipetted into test tubes. To each tube was added 0.1 ml bacteria or LPS suspended in the same buffer as used in the phase system. The tubes were inverted repeatedly for mixing and the phases were allowed to settle for 20 min. Then 0.5 ml samples were withdrawn from each phase. The radioactivity was measured in an auto-gamma scintillation counter (Packard) and the distribution of bacteria or LPS to both the top and bottom phase and to the interface was calculated.

Counter-current distribution An automatic thin-layer counter-current distribution (CCD) apparatus (Incentra Research and Development AB Stockholm, Sweden) with 60 cavities was used (1) All except cavity 1 and 31 were charged with 0.50 ml bottom phase and 0.70 ml top phase. Samples suspended in 1 30 ml of the complete phase system were added to cavity 1 and 31 In this way two samples were analysed concurrently and the experimental variation between different experiments was reduced. After 29 transfers, each with a shaking time of 40 and settling time of 5 min, 1.5 ml of buffer was added to each cavity to break the phases. The cavities were emptied into separate vials and the radioactivity determined.

Electrophoresis. Free zone electrophoresis of bacteria and LPS was performed in a Hjertén apparatus, i.e. the electrophoresis chamber was horizontal quartz tube, slowly rotating around its long axis (6) The material distribution of withdrawn fractions was determined by scanning in ultraviolet light and/or by carbohydrate analysis by the anthrone method

RESULTS

Influence of surface LPS structure on partitioning It has been shown earlier that the partition of *S typhimurium* 395 MS differs widely from that of *S typhimurium* 395 MR10 (16) The partition of their LPS was similar to the partition of the correlated strains. Thus, the S bacteria and the S LPS were recovered from tubes with higher numbers as a consequence of affinity for the PEG-rich phase, whereas the R bacteria and the

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Partition in a dextran-PEG two-phase system containing positively (trimethylamino-) or negatively (sulphonate) charged PEG and free zone electrophoresis discerned a substantially greater negative surface charge on MR10 than on MS bacteria and LPS.

MATERIALS AND METHODS

Bacteria. The smooth, mouse-virulent *Salmonella typhimurium* 395 MS and LT2, and the mutants derived from them has been described earlier (11). All strains were kept at 4 C on agar slants before use. Bacteria were grown, harvested and heat killed as described previously (15). The uridine diphosphate (UDP)-galactose(gal)-4-epimerase-less mutant, LT2 M1 was cultivated according to *Lindberg et al.* (10) in glucose medium in the absence of galactose yielding LT2 M1 (glu) or in the presence of galactose giving LT2 M1 (gal).

LPS preparation. LPS was extracted from S bacteria (MS LT2 M1 grown with galactose) by the phenol/water method described by *Westphal et al.* (21) and from R bacteria (MR10, LT2 M1 grown without galactose) by the phenol/chloroform/petroleum ether method according to *Galanos et al.* (5).

Labelling of bacteria and LPS. The bacteria were labelled with ^{51}Cr as described previously (15). The LPS was labelled with ^{51}Cr in the following way (2). LPS was suspended in distilled water to concentration of 5 mg/ml. 7 ml of this suspension was mixed with 0.5 ml $Na_2{}^{51}CrO_4$ (0.5 mCi) and incubated at 37 C. After 24 h the mixture was dialysed against distilled water until no significant activity could be detected in the dialysate. Finally the labelled LPS was concentrated to approximately 10 mg/ml.

Phase systems. A two-phase system (1) was prepared from stock solutions of 20 per cent PEG 6000 (Carbowax 6000, Union Carbide, New York, N.Y.), 20 per cent dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden), 0.1 M tris-(hydroxymethyl)aminomethane (tris) buffer pH 7.0 and distilled water and was allowed to equilibrate at 4 C overnight. The total system contained 4.4 per cent (w/w) PEG and 6.2 per cent (w/w) dextran in 0.05 M tris buffer (pH = 7.2). In some experiments the influence on partition by charged PEG was tested by substituting part of the PEG with charged PEG (8). Positively charged trimethylamino($(CH_3)_3N^+$–)-PEG called TMA-PEG and negatively charged sulphonate-(SO_3^-–)-PEG called S-PEG, were kindly supplied by Dr. G. *Johansson* Umeå, Sweden.

Partition analysis. Single step. Two ml of bottom phase and two ml of top phase were pipetted into test tubes. To each tube was added 0.1 ml bacteria or LPS suspended in the same buffer as used in the phase system. The tubes were inverted repeatedly for mixing and the phases were allowed to settle for 20 min. Then 0.5 ml samples were withdrawn from each phase. The radioactivity was measured in an auto-gamma scintillation counter (Packard) and the distribution of bacteria or LPS to both the top and bottom phase and to the interface was calculated.

Counter-current distribution. An automatic thin-layer counter-current distribution (CCD) apparatus (Incentive Research and Development AB, Stockholm, Sweden) with 60 cavities was used (1). All except cavity 1 and 31 were charged with 0.50 ml bottom phase and 0.70 ml top phase. Samples suspended in 1.30 ml of the complete phase system were added to cavity 1 and 31. In this way two samples were analysed concurrently and the experimental variation between different experiments was reduced. After 29 transfers, each with a shaking time of 40 s and a settling time of 5 min, 1.5 ml of buffer was added to each cavity to break the phases. The cavities were emptied into separate vials and the radioactivity determined.

Electrophoresis. Free zone electrophoresis of bacteria and LPS was performed in a Hjertén apparatus, i.e. the electrophoresis chamber was a horizontal quartz tube, slowly rotating around its long axis (6). The material distribution of withdrawn fractions was determined by scanning in ultraviolet light and/or by carbohydrate analysis by the anthrone method.

RESULTS

Influence of surface LPS structure on partitioning. It has been shown earlier that the partition of *S. typhimurium* 395 MS differs widely from that of *S. typhimurium* 395 MR10 (16). The partition of their LPS was similar to the partition of the correlated strains. Thus, the S bacteria and the S LPS were recovered from tubes with higher numbers as a consequence of affinity for the PEG-rich phase, whereas the R bacteria and the

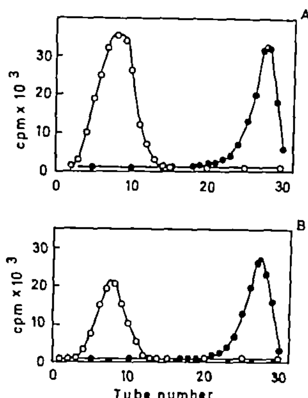


Fig 1 Counter-current distribution (?9 transfers) of *S typhimurium* 395 MS (●) and 395 MR10 (○) bacteria (A) and their respective lipopolysaccharides (B) The cells and LPS were labelled with ^{51}Cr

R LPS were recovered from the earlier tubes as a consequence of affinity for the dextran-rich phase (Fig 1) The partition of LPS from a UDP-galactose-4-epimeraseless mu tant, LT2 M1 was determined by the com position of the growth medium (Fig 2) Thus, cultivation of bacteria in the absence of galactose which did not allow synthesis of S-specific LPS made the partition of both the bacteria and their LPS similar to that of R10 By adding galactose to the growth me dium, which made possible synthesis of the S-specific repeating unit, the partition of bac teria and LPS became more similar to MS.

Partition of bacteria and corresponding LPS in the presence of charged PEG The presence of TMA PEG or S-PEG in different concentrations (6.25 and 12 5 per cent (w/w)) did not influence the partition of MS bacteria (Fig 3) or LPS (Fig 4) In contrast, R10 bacteria (Fig 3) and LPS (Fig 4) were gradually moved towards the top phase by increasing concentrations of TMA PEG whereas the partition was little influenced at either concentration of S-PEG

Effect of pH on partition Variation of pH between 2.5 and 8.5 produced no measurable change in the partition of MS bacteria and LPS (Table 1) At pH ranging between 8.5 and 3.5 the partition of R10 bacteria and LPS was little affected. Further reduction of the pH to 2.5 enhanced the effect. However only 27 per cent of the bacteria and 59 per cent of the LPS were in the bottom phase as compared to 79 and 93 per cent respectively at pH = 8.5

Electrophoresis of bacteria and LPS Free zone electrophoresis (6) was performed in tris-HAc buffers of the same ionic strength but with different pH Typical scanning diagrams from LT2 M1 (gal) bacteria and LPS are reproduced in Fig 5a and 5b, respectively Most electrophoretic diagrams with bac teria showed more than one peak. However it was easy to decide which peak(s) corre-

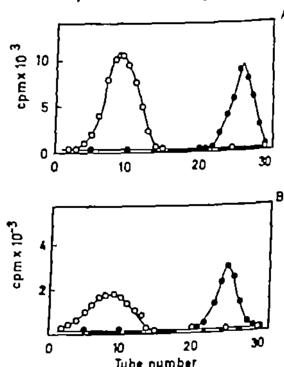


Fig 2 Counter-current distribution (29 transfers) of a DUP-gal-4-epimeraseless mutant (LT2 M1) grown 4 h in medium without galactose (○) and with galactose (●) (A) and their respective LPS (B) The cells and LPS were labelled with ^{51}Cr

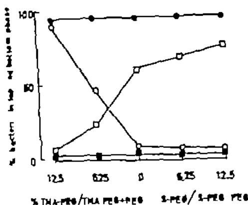


Fig. 3. Partition of bacteria, *Salmonella typhimurium* 395 MR10 (open symbols) and MS (filled symbols) in the presence of different concentrations of TMA-PEG and S-PEG. ○ = top phase, □ = bottom phase. The cells were labelled with ^{51}Cr.

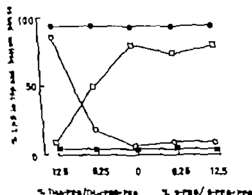


Fig. 4. Partition of LPS. *Salmonella typhimurium* 395 MR10 (open symbols) and MS (filled symbols) in the presence of different concentrations of TMA-PEG and S-PEG. ○ = top phase, □ = bottom phase. The LPS was labelled with ^{51}Cr.

ponded to bacteria owing to their opalescence. The other peak(s) in the bacterial sample represented non-cellbound material. The mobilities were calculated as described earlier (6). They are assembled in Table 2. The calculations demonstrate clearly that the R bacteria had a much greater mobility towards the anode than the corresponding S bacteria, thus presenting an argument for a greater

TABLE 1. *Partition of S. typhimurium 395 MS and R10 Bacteria and LPS in Aqueous Two-phase Systems*) *at Different pH*

pH	Per cent bacteria and LPS in bottom phase: Bacteria MS	Bacteria MR10	LPS MS	LPS MR10
2.5	8	27	4	59
3.5	7	64	4	90
4.5	7	68	4	96
5.5	7	77	4	98
6.5	7	76	4	93
7.5	7	78	4	95
8.5	7	79	5	93

) Phase composition: 6.28 per cent (w/w) dextran, 4.4 per cent (w/w) PEG, 0.1 M NaCl, and 0.05 M citrate-phosphate buffer (2.5–7.5) or 0.05 M tris buffer (7.5–8.5).

negative surface charge. The mobility was increased by augmenting the pH from 5.5 to 7.0 and 8.6. With the LPS preparations, UV scanning was complemented by carbohydrate analysis of withdrawn fractions for the localization of the peaks. Fig. 5b shows that the LPS was heterogenous and contained material of both carbohydrate and proteinaceous nature.

DISCUSSION

Investigations of *S. typhimurium* 395 MS and a series of mutants derived from it and differing by their surface LPS, have shown a relationship between physico-chemical surface properties, as assessed by aqueous biphasic partitioning (13, 16) and phagocytosis (15), resistance to intracellular bactericidal systems (18) and attachment of the bacteria to HeLa cells *in vitro* (9). In the present experiments, which were aimed at elucidating surface charge characteristics, the partition of bacteria and LPS was very similar. Thus, in a multistep partition system with dextran and PEG in the CCD-apparatus (Fig. 1), the partition ratio (1) of R bacteria was 0.38 (peak in tube 8), which is identical with that of R LPS. The peak of S bacteria was recovered in tube 28 and that of S LPS in tube 27. Further experiments with pheno-

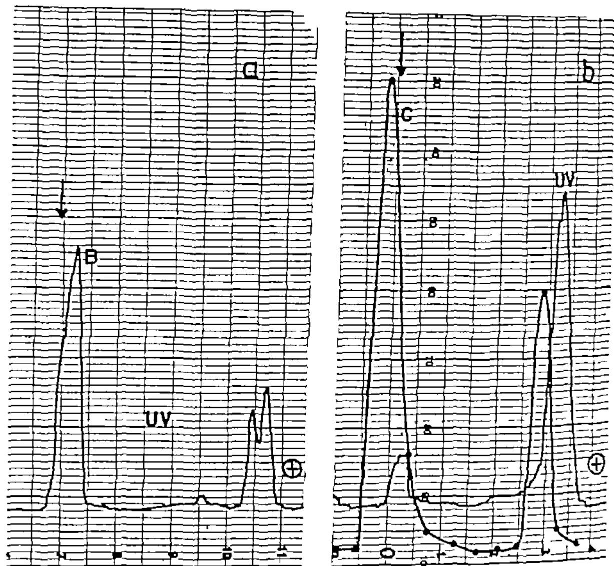


Fig 5 Typical electrophoretic diagrams of LT2 M1 (gal) bacteria determined by UV-scanning (a) and of LPS determined by UV-scanning and carbohydrate analysis (b) B indicates bacteria and C carbohydrate ↓ starting point and ⊕ direction towards anode.

Table 2 *Mobilities) of* Salmonella typhimurium *Bacteria b) and Lipopolysaccharides) at 12° C in 0 05 M Tris HAC Buffers (μ d) = 0 05)*

pH	Bacteria				LPS	
	MS	MR10	LT2 M1 (glu)	LT2 M1 (gal)	LT2 M1 (glu)	LT2 M1 (gal)
5.5	0.3	16	7 7	1.5	{ 0 27	N.D)
7 0	1 4	14	9.8	4 0	N.D	N.D
8 6	1 8	17	12	2 0	{ 1 4 22	{ 0 18

) In -10^{-4} $cm^2 sec^{-1} volt^{-1}$
b) Peaks determined by UV absorption.
) Peaks determined by carbohydrate analysis.
d) μ = Ionic strength.
) N.D = Not determined

typical R and S bacteria of the same UDP gal-4-epimeraseless mutant, LT2 M1 gave analogous results (Fig 2) These results confirm that the surface LPS influences decisively the physico-chemical surface properties.

By including charged PEG in the dextran-PEG system, charge effects at partitioning are amplified. Particles of opposite charge to the substituted PEG are attracted to the top phase, whereas those of the same charge are repelled (1 8) R bacteria (Fig. 3) and R LPS (Fig 4) were moved towards the top phase by TMA PEG which shows that they carry negative surface charge. S bacteria and S LPS were not influenced by either charge, thus indicating no net charge.

The inertia of S bacteria against charged polymers is in agreement with the data in Table 2, which shows that S bacteria have a considerably lower mobility i.e. a smaller surface charge density than the R bacteria. The electrophoretic results refer primarily to the charge located within the electrical double layer which at the ionic strength used has a "thickness of about 10 Å (20) In contrast, partition in phase systems is considered dependent on charge deeper in the cell envelope (3) Thus, the absence of charge effects on S bacteria and S LPS in the phase systems indicates a fairly deep uncharged zone in these bacteria. This zone might correspond to the S specific polysaccharide chains of the LPS.

Furthermore, the difference in the mobilities of the S bacteria 395 MS and LT2 M1 (gal) together with the discrepancy in the pH dependence of their mobilities, indicate that also the two S bacteria may have different surface structures. The same applies to the R bacteria 395 MR10 and LT2 M1 (glu)

In Fig 5b two UV-peaks and two carbohydrate peaks can be seen. Similar heterogeneity was found in most of the lipopolysaccharides examined, and in many experiments the UV and carbohydrate peaks did not coincide This makes it difficult to find a clear relationship between the mobilities of the lipopolysaccharides and the bacteria. The electrophoretic heterogeneity of LPS observed may however be only apparent. The different peaks might correspond to free LPS, to micelles of LPS or to a precipitate of LPS.

It should be noted that for some bacteria and lipopolysaccharides the mobility did not increase simultaneously with an increase in pH (Table 2) This observation indicates that anions are desorbed from the cell surface and/or cations adsorbed when pH increases or that the surface structure is altered in such a way that anions are hidden and/or cations exposed. As regards the hydrophobicity of the bacteria, it was proposed that the complete LPS of for instance, MS bacteria was hydrophilic and blocked more hydrophobic regions, primarily the lipid A (15) The present results suggest a similar function also with respect to charge interaction. However they do not exclude the influence of other envelope constituents. Basal-core phage-receptors have actually been found also in S strains although in small quantities (12) The greater electrophoretic mobility of LT2 M1 (gal) than of MS may also reflect an incompleteness of the surface layer of LT2 M1 Phages that use outer membrane proteins as their receptors have also been shown to interact to a greater extent with strains of *Salmonella typhimurium* that lack the complete O-antigen (14) In the LPS the potentially negatively charged groups are the core 2-deoxyoctyonate (KDO) and phosphate groups linking different PS chains. However since the preparations of the different LPS were not free from UV absorbing material, an influence of protein cannot be excluded.

To conclude, hydrophobicity (13 19) and negative charge (present investigation) seem to characterize the surface of R bacteria *which are liable* to contact with different host cells, whereas hydrophilicity and absence of charge on S bacteria work against an interaction.

However the relation between negative charge and hydrophobicity may be rather subtle. If the negative charge is introduced superficially to the LPS for instance as the K-antigen of certain *Escherichia coli*, it

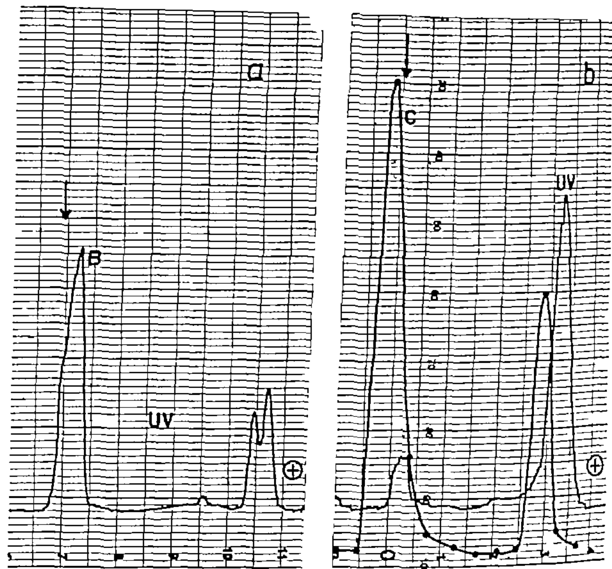


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seems to increase resistance to phagocytosis (unpublished observation) The precise mechanisms of this behaviour remain to be clarified.

The technical assistance of *Kerstin Hagersten* and *Irja Blomqvist* is gratefully acknowledged.

This investigation was supported by the *Swedish Medical Research Council* (Grant B76-16X-02183-10) and the *Swedish Natural Science Research Council*

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INTERACTION OF HYDROXYAPATITE AND PROTEIN-COATED HYDROXYAPATITE WITH *STREPTOCOCCUS MUTANS* AND *STREPTOCOCCUS SANGUIS*

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Rölla, G., Robrish, S. A. & Bowen, W. H. Interaction of hydroxyapatite and protein-coated hydroxyapatite with *Streptococcus mutans* and *Streptococcus sanguis*. Acta path. microbiol. scand. Sect. B, 85 341–346, 1977

The present study showed that *S. mutans* and *S. sanguis* behaved like negatively-charged particles in their interaction with hydroxyapatite in vitro. Phosphate in the system inhibited bacterial uptake by apatite, whereas calcium increased the uptake. A layer of acidic protein inhibited the uptake of bacteria by hydroxyapatite. The opposite was true when basic protein was first adsorbed to the apatite. A saliva film on the apatite decreased the uptake of bacteria, supporting the view that acidic proteins are selectively adsorbed by hydroxyapatite from saliva. The results indicate clearly that electrostatic forces may be involved in bacterial interaction with tooth surfaces.

Key words: Streptococcus; hydroxyapatite; adsorption; dental plaque; dental disease.

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Received 19.iv.77 Accepted 28. 77

The characteristic composition of dental plaque has prompted many recent investigations on possible specific mechanisms of adherence. These studies have been reviewed exhaustively by *Gibbons & van Houte* (1975). While specific adherence factors have been demonstrated and may be important, there are also conceivable non-specific mechanisms of bacterial attachment to a tooth surface coated with pellicle protein. Recently such a non-specific mechanism has been outlined based on the amphoteric nature of hydroxyapatite, the ionic nature of pellicle protein and the negative charge on the surface of bacteria (*Rölla* 1976, 1977). The general theory for this ion exchange model has been developed by *Bernardi et al.* (1972) who used hydroxyapatite columns for the separation of proteins. This paper presents data to examine the nature of the binding of *S. mutans* and *S. sanguis* to hydroxyapatite. The effect of adsorbed protein on the binding of the organisms to hydroxyapatite particles has also been studied.

MATERIALS AND METHODS

Organisms

The following strains of streptococci maintained as stock cultures in our laboratory were used in this study: *S. sanguis* 10558 and *S. mutans* 6715 and LM7. Organisms were grown to stationary

phase in a N_2-CO_2 (95 5) atmosphere at 37 C in Jordan s streptococcal growth medium and Brain Heart Infusion (Difco)

Carbon 14 was incorporated into the cells by growth in a medium containing labelled amino acids obtained from an algal protein hydrolysate Fifty microlitres (2.5 microcuries) of a sterile uniformly C-14 labelled algal protein hydrolysate amino acid mixture (Amersham/Searle) was added aseptically to 100 ml of liquid medium in a 250 ml Erlenmeyer flask The broth was inoculated with the organism and incubated at 37 C in a New Brunswick Aquatherm shaking water bath in an N_2-CO_2 (95 5 per cent) atmosphere until the cells were in stationary phase The cells were sedimented by centrifugation in an International clinical centrifuge and the old medium discarded The bacterial sediment was then made up in 100 ml of fresh medium and allowed to grow to stationary phase The cells were harvested by centrifugation and the cell mass washed with distilled water This was repeated three times, yielding a supernatant liquid which was no more than twice the background count when assayed with the acid of a Packard Tri-Carb liquid scintillation counter Dry weight of bacteria was calculated from the turbidity of the suspension determined at 550 nm in 1 mm cells in a Gilford 2400 spectrophotometer These data were compared with dry weight/turbidity relation previously calculated for *S mutans* and *S sanguis* Because the organisms were grown in similar conditions, the cell yields were similar and corresponded to about 7 3 micrograms dry weight of organism per microlitre of bacterial suspension.

Reagents

Protamine phosphate was from Sigma bovine albumin fraction V from Armour and hydroxylapatite Bio Gel HTP from Bio-Rad. Saliva was collected in a test tube from one of us (G R.) and used in these experiments without further treatment

Assay

100 mg samples of hydroxyapatite were weighed into 16 × 150 mm test tubes and the appropriate proteins or samples of saliva were added in 3 ml of distilled water The mixture was agitated with a Vortex mixer and allowed to stand at room temperature (25 C) for 10 min. The hydroxyapatite was sedimented by centrifugation in an international clinical centrifuge The supernatant fluid was decanted from the packed sediment and discarded The bacterial suspension was then added in a 2 or 3 ml total volume of diluent as indicated, and the hydroxyapatite and bacterial mixture were resuspended by agitation with the aid of a Vortex mixer This mixture was allowed to stand at room temperature for 30 minutes, at which time the hydroxyapatite had fallen out of suspension. When adsorption was determined in the presence of inorganic ions alone the organisms and diluent were added to the 100 mg hydroxyapatite sample and the mixture agitated, and the hydroxyapatite was then allowed to fall out of suspension at room temperature for 30 minutes. One ml aliquots of the organisms left in suspension were removed and transferred to scintillation vials containing 10 ml of Packard "Insta-Gel" for assay of C-14 using a liquid scintillation counter When aliquots of cells were allowed to stand at room temperature for 30 minutes, there was no detectable loss in radioactivity in the bulk solution due to settling of some portion of the bacterial suspension.

RESULTS

Preliminary experiments confirmed that a positive correlation existed between the surface area (or weight) of the apatite and the number of bacteria adsorbed. One hundred mg of apatite was found to be suitable for the volume and concentrations of bacteria chosen and the time of interaction allowed All the experiments were performed with this amount of hydroxyapatite and three different concentrations of bacteria. All data compared in any one of our figures were obtained in one series of experiments performed at the same time with identical batches of bacteria. The specific activity of the bacteria was usually 3 to 5 × 10^3 c p.m per ml, when diluted in 10 ml of scintillation liquid.

Fig 1 shows the effect of inorganic ions on the adsorption of glucose-grown *S mutans* to hydroxyapatite. Calcium increased the uptake of *S mutans* markedly whereas the anions fluoride and phosphate decreased the uptake to about 50 per cent of the potentially adsorbable cells. When 50 mM of phosphate was present during the adsorption process, almost no uptake of bacteria was observed. Calcium, phosphate and fluoride showed a similar influence on the adsorption of *S sanguis* in identical experiments. About 50–70 per cent of the available bacteria were usually taken up by the apatite in our model system under normal conditions, as exemplified by the water control in Fig 1 In contrast when sucrose-grown *S mutans* was used with untreated hydroxyapatite an up-

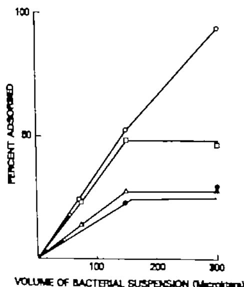


Fig 1 Effect of floride, calcium and phosphate on the adsorption of aliquots of labelled *S mutans* to 100 mg amounts of hydroxyapatite. Organisms adsorbed in 3 ml total volume of water suspension (□) in the presence of 10 mM $CaCl_2$ (○) 10 mM NaF (●) and 10 mM phosphate buffer (Δ) Activity of labelled bacteria 2393 counts per 300 microlitre volume of bacteria. Counts adsorbed are expressed as fraction of the 300 microlitre total.

take of more than 90 per cent of the bacteria was observed (Figs. 2 and 4) and many more cells were adsorbed in the presence of phosphate (Fig 2) thus indicating a stronger affinity for hydroxyapatite by sucrose-grown *S mutans*

The effect of different films adsorbed to the apatite surface on subsequent uptake of *S mutans* is shown in Figs. 3 and 4 A film of the basic protein, protamine, increased the uptake of *S mutans* whereas a similar albumin film decreased the uptake markedly Saliva treatment influenced the uptake in the same way as albumin (Fig 3) The concentrations of proteins in the solutions during pretreatment were comparable with that of saliva The presence of 0 5 M of NaCl during the adsorption decreased the uptake of sucrose-grown bacteria by saliva-treated hydroxyapatite (Fig 4) The uptake of *S sanguis* by protein-treated apatite showed the same pattern as described for *S mutans*.

DISCUSSION

A model consistant with all the data presented in this paper has been proposed by *Rölla* (1976 1977) to describe the sequence of events in the adherence of bacteria to the tooth surface The essence of the model is that acidic proteins are adsorbed to the amphoteric hydroxyapatite surfaces of the enamel. This protein surface or pellicle then binds the negatively-charged bacterial surface by means of calcium bridges. Certain predictions can be made from the model about the effect of various anions on the adsorption of proteins to hydroxyapatite particles and the subsequent binding of bacteria to these composite surfaces. The adsorption of bacteria to the surface will depend on the relative cationic nature of the hydroxyapatite

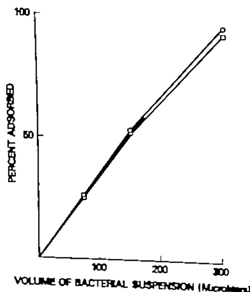


Fig 2 Effect of phosphate on sucrose-grown *S mutans* (LM7) The bacteria were grown in the presence of sucrose, as described in the methods. Bacteria adsorbed to 100 mg batches of hydroxyapatite in water (○) and in presence of 10 mM phosphate buffer (□) Activity of 300 microlitre volume (100 per cent) of cells was 5600 counts per s.

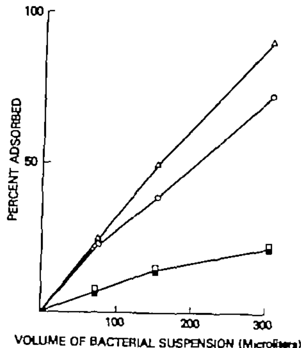


Fig 3 Effect of ions and proteins on the adsorption of *S mutans* (LM7) to hydroxyapatite. Cell concentration and assay as described in the methods *Proteins Protamine phosphate and bovine* serum albumin 1 mg/ml Saliva was from one of us (G R.) The 100 mg hydroxyapatite was pretreated with 3 ml of the protein solution or water for the concentrations described in the method and assay performed after hydroxyapatite particles had settled The activity of cells was 2057 counts per s in 300 microlitres = 100 per cent. (Untreated apatite (○) protamine-treated (△) albumin treated (□) saliva-treated (■)

surface (or rather of the hydration shell covering the surface) and the anionic nature of the bacterial surface. Free anions will tend to compete with the negatively-charged bacteria Acidic protein bound to the hydroxyapatite surface will reduce the cationic nature of the surface and suppress binding of bacteria while basic proteins should enhance the cationic nature of the surface and increase *the amount of binding* The data presented in this paper conform to the model in all respects.

In the case of glucose-grown cells, the presence of phosphate or fluoride as common anions effectively suppressed the binding of *S mutans* and *S sanguis* to the hydroxyapatite. The anionic character of the surface of *S mutans* could be enhanced by growing the organisms *in the presence of sucrose.* This has been demonstrated by studies on the binding of *glucose-grown and sucrose-grown* cells of *S mutans* to ion exchange materials and by direct observation of the electrophoretic properties of the polysaccharide from sucrose-grown cells (*Keldrup & Fauda Nielsen* 1972) Our studies confirmed these observations. When *S mutans* was grown in the presence of sucrose, forming polysaccharidecoats on the cells, its anionic character was strong as seen by the continued adsorption of the cells to a hydroxyapatite surface in the presence of the phosphate anion. This concentration of phosphate was sufficient to suppress significantly the adsorption of glucose-grown cells to the hydroxyapatite surface. Phosphate has been shown to suppress the adsorption of Lpoteichoic acid extracted from *S mutans* to the surface of

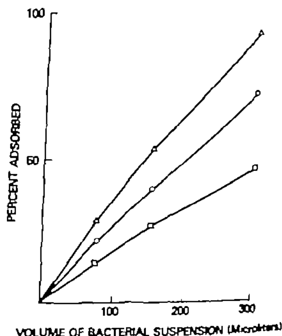


Fig 4 Effect of NaCl on the adsorption of sucrose-grown *S mutans* (6715) to saliva-treated apatite *For the samples indicated, 100 mg of hydroxyapatite was treated with 3 ml saliva* After sedimentation as described and decantation of the saliva the samples were made to 3 ml volume containing cells plus distilled water (□) and 0.5 mM NaCl (○) A control containing untreated hydroxyapatite was included (△) The activity of the cells was 2921 counts per s in 300 microlitres = 100 per cent.

hydroxyapatite (*Ciardi et al.* 1977). Lipoteichoic acid is presumably the major factor in the strength of the anionic character of the surfaces of *S. mutans* and *S. sanguis* and the coat of the sucrose-grown *S. mutans* is known to contain high amounts of this substance, thus rendering increased charge to the cell (*Melvaer et al.* 1974, *Kelstrup & Funder Nielsen* 1972). The marked influence of the anions and cations on the adsorption of bacteria by hydroxyapatite demonstrates that the buffer used may be crucial in adsorption experiments *in vitro* and *in vivo*. Some of the present confusion in this field in the literature is undoubtedly caused by neglect of this factor.

The results presented in this report are in agreement with those reported by *Ericson et al.* (1975) who also showed, using turbidity measurements, that the adsorption of a suspension of *S. mutans* was inhibited when hydroxyapatite particles were pretreated with saliva. The more sensitive assay of adsorption of radioactivity labelled bacteria was used by *Liljemark & Schauer* (1975), who observed that some strains of oral streptococci were adsorbed more completely to saliva-coated hydroxyapatite than to uncoated hydroxyapatite. The disparity between the results we are reporting and those reported by *Liljemark & Schauer* can presumably be explained by the high phosphate content (0.067 M) in their system.

The characteristics of the adsorption of protein to hydroxyapatite surfaces and the effects of various ions have been investigated by *Bernardi et al.* (1972). Their studies showed that calcium ions were effective in eluting basic proteins from hydroxyapatite column, while these ions were ineffective in the elution of acidic proteins. In contrast, phosphate in low concentrations was effective in eluting acidic proteins from hydroxyapatite. In our current studies, adsorption of *S. mutans* cell was suppressed when the hydroxyapatite particles were pretreated with an acidic protein, albumin, or with saliva which also coats the hydroxyapatite particles with acidic proteins. As would be expected, the pretreatment of hydroxyapatite with a basic protein, protamine, enhanced the cationic properties of the hydroxyapatite surface and bacteria were bound more effectively. The effect of the adsorbed saliva film corroborated with the view that acidic proteins are selectively adsorbed to hydroxyapatite from saliva (*Sönju & Rölla* 1973, *Rölla & Embery* 1977). The experiment illustrated in Fig. 4 showed that the binding of bacteria to the adsorbed saliva protein (pellicle) was reduced by the presence of 0.5 mg NaCl thus supporting the ionic character of this interaction (*Rölla* 1971).

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Acta path. microbiol. scand. Sect. B, 85 347–349 1977

TREATMENT OF *MYCOPLASMA HYORRHINIS* CONTAMINATED TISSUE CULTURES WITH A MIXTURE OF ANTIBIOTICS

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Ulrich, K. & Briand, P. Treatment of *Mycoplasma hyorrhinis* contaminated tissue cultures with a mixture of antibiotics. Acta path. microbiol. scand. Sect. B, 85 347–349 1977

Results obtained using a combination of antibiotics to control mycoplasmas in tissue cultures are described. Cell strains and established cell lines from several mammalian species grown in tissue culture were found to be highly contaminated with *M. hyorrhinis*. Cultures were treated with a mixture of three antibiotics consisting of gentamicin, tetracycline and chloramphenicol, and since that time tests for mycoplasmas in the treated cultures have consistently yielded negative results. Apart from a transient cytostatic effect on the cells during the treatment, no apparent unwanted effects were observed. The mixture of three antibiotics appeared to be superior to treatment with antibiotics singly or combinations of two antibiotics.

Key words: Mycoplasmas; tissue culture; antibiotics.

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Received 3. 77 Accepted 8.vii.77

Mycoplasma contamination of tissue cultures often presents a serious problem because the microorganisms may preclude many investigations by interfering with a number of cell functions (3, 9). The problem becomes particularly serious if the cultures are irreplaceable, in which case reliable methods for the suppression of mycoplasmas in the cultures are needed. A variety of methods, many of which depend on the addition of antibiotics to the cultures, have been described (1, 9).

Since a widespread contamination with mycoplasmas was found in most cell lines carried in our laboratory, it became mandatory to institute an efficient treatment to control the infection. The present paper describes the results obtained with a mixture of three antibiotics.

MATERIALS AND METHODS

Cells

The tissue cultured cells included in the present study originated from mouse, hamster, cat and human tissues. Malignant as well as normal adult and embryonal tissues are represented.

Routine stock cultures of cells are grown as monolayers in Eagle' MEM (2) or in modified MEM, Fib 41B (6) supplemented with 5 or 10 per cent heat-inactivated fetal bovine serum and without antibiotics. Trypsin which is used for detachment of cells is irradiated with 5 Mrad before use.

Detection of Mycoplasmas

Mycoplasmas were detected by demonstrating growth of characteristic colonies on agar according

to the method described by *Hayflick* (4) An 0 2 ml sample of supernatant medium from the suspected tissue culture was seeded onto agar (standard 5 cm Petri dishes with 8 ml of agar medium) and in 4 ml of broth medium (3) After three days of incubation broth cultures were streaked on agar and also reseeded in broth. This procedure was repeated once more. All agar cultures were incubated for nine days in humidified, atmospheric air at 37 C Agar plates were examined for colonies every three days In one instance, a 'large specimen broth culture procedure (1) was used, incubating the total volume (4 ml) of supernatant medium with 80 ml of broth as the initial step in the mycoplasma test.

Identification of the mycoplasma species involved was kindly carried out by Dr *K Lind* the Mycoplasma Laboratory Statens Seruminstitut, Copenhagen, using the metabolic inhibition test as described by *Taylor Robinson et al.* (10)

Treatment with Antibiotics

The following antibiotics were used for the treatment of contaminated tissue cultures Oxytetracycline (O) 25 μg/ml, neomycin B (N) 500 μg/ml kanamycin (K) 200 μg/ml gentamicin (G) 100 μg/ml tetracycline (T) 10 μg/ml and chloramphenicol (C) 5 μg/ml. The antibiotics were used either singly or in different combinations added to the medium in the final concentrations given above (7) For two weeks the medium was renewed three times weekly and each time freshly prepared antibiotics were added. After a further cultivation period of one to four weeks in antibiotic free medium, the cells were tested for mycoplasmas.

RESULTS

Samples selected at random from the contaminated cell lines showed the presence of *M hyorrhinis* Treatment with the combination of antibiotics, gentamicin tetracycline and chloramphenicol (GTC) resulted in the apparent elimination of mycoplasmas from all cell lines included in the study (Table 1) In one case the GTC-treatment had to be repeated before the mycoplasma test became negative, whereas in another case mycoplasmas could not be demonstrated after treatment with GC only Neither treatment with HK ON nor O alone was capable of eliminating mycoplasma infection in the cultures. An infected and untreated cell line STL-4 which served as positive control in our mycoplasma test invariably gave rise to growth of mycoplasma colonies.

TABLE 1 *Cultivation of Mycoplasmas from Cell Lines after Treatment with Different Antibiotics*

Antibiotics b	No. of cell lines + mycoplasmas	No. of cell lines — mycoplasmas
NK	1	0
ON	1	0
O	4	0
GC	0	1
GTC	1	14

a. Different established cell lines of mouse, hamster and cat origin as well as human embryonal cell strains.
b. Chloramphenicol (C) 5 μg/ml, Gentamicin (G) 100 μg/ml, Kanamycin (K) 200 μg/ml, Neomycin B (N) 500 μg/ml, Oxytetracycline (O) 25 μg/ml and Tetracycline (T) 10 μg/ml final concentration in the cell culture medium. Antibiotic treatment was given for a period of 14 days.

TABLE 2 *Cultivation of Mycoplasmas from a Contaminated Cell Line (STL-4) after Treatment with Different Antibiotics either Singly or Combined*

Antibiotics Day 1–14	Cultivation of mycoplasmas Day 21	Day 49	Day 70
None	+	+	+
G	+		
TC	+		
GTC	—	—	—b

a. Chloramphenicol (C) 5 μg/ml, Gentamicin (G) 100 μg/ml and Tetracycline (T) 10 μg/ml final concentration in the cell culture medium
b. Large specimen broth culture procedure

All cell lines were stored in liquid nitrogen after a negative mycoplasma test was obtained. Four of these cell lines were thawed and retested after growth in antibiotic free medium for various length of time ranging from 21 to 116 transfers. Mycoplasmas could not be demonstrated by cultivation in any of the four cell lines.

The efficiency of the treatment with GTC was further established by treating the infected STL-4 cell line with G alone or with

T and C together to compare treatments. Only the GTC treated culture became mycoplasma-negative and remained so when tested after 35 and 56 days of cultivation (Table 2).

DISCUSSION

A number of cell lines in our laboratory were contaminated with mycoplasmas. This was evidenced by cytopathic effects (CPE) such as unusual acidification of the culture medium and rounding up and detachment of the cells. Mycoplasmas were demonstrated in these cell lines by cultivation of typical mycoplasma colonies on agar. The only mycoplasma species found in the cell lines was *M. hyorrhinis*. Despite reports that *M. hyorrhinis* may be difficult to cultivate (8), demonstration of the mycoplasmas by growth in broth and agar worked very reliably.

Our results clearly indicate that gentamicin, tetracycline and chloramphenicol in combination are superior to a single antibiotic or even a combination of two antibiotics. After GTC-treatment of all mycoplasma contaminated cell lines, CPE disappeared and mycoplasmas could not be demonstrated by cultivation at repeated occasions even after prolonged cultivation of the cell lines in antibiotic free medium.

During the described treatment with GTC, the number of cells in the cultures usually became stationary as indicated by lack of need for subculturing. However, no apparent cell loss was ever observed and cultures always resumed normal growth immediately after termination of the treatment. In addition, no adverse effects were observed on cell morphology, growth patterns, plating efficiencies or virus multiplications in cell lines that had undergone treatment.

The authors wish to acknowledge the excellent technical assistance of *Marianne Hansen*, *Ulla Beckh* and *Birthe Antonsen*.

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Acta path. microbiol. scand. Sect. B 85 350 1977

BRIEF REPORTS

SINGLE-CELL PROTEIN AS A BACTERIAL SUBSTRATE

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Fossum, K. & Almlid, T Single-cell protein as a bacterial substrate. Acta path. microbiol. scand. Sect. B, 85 350 1977

It was found that the pathogenic and potentially pathogenic bacteria tested were able to grow and produce extracellular enzymes and toxins in substrates based on extract from various products of Single-cell protein (SCP) as well as in suspensions of SCP In addition to the hygienic aspects of these findings the possibility of utilizing SCP as substrate for cultivation of bacteria is pointed out.

Key words Single-cell protein bacterial substrate toxin production.

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Received 8.ix.77 Accepted 20.ix.77

The question of whether pathogenic, and potentially pathogenic, bacteria are able to grow and produce toxins in substrates consisting of Single-cell protein* (SCP) has arisen with the introduction of SCP as an animal feedstuff The possibility exists that the water activity and the temperature of such products can, unintentionally be favourable for a sufficient length of time to allow such growth

In the present work peptone and meat extract in various culture media were replaced with extracts of several relevant commercial SCP products of bacterial and fungal origin The extracts were obtained by boiling the various SCP products in water followed by centrifugation and filtration Sterilized suspensions of the SCP products were also used as substrates. The substrates were made so as to contain 1.5–2 per cent protein. The bacteria tested (*Clostridium perfringens Escherichia coli Pseudomonas aeruginosa Salmonella texas* and *Staphylococcus aureus*) all grew in liquid media and on agar media with the SCP-extracts as the only nutrient, and also in suspensions of the SCP-products. The growth rates differed somewhat, but were, in some SCP products, similar to the controls. Bacteria producing extracellular enzymes such as amylases DNases, haemolysins, lipases and proteinases also gave rise to these enzymes when grown in SCP-substrates. The production of toxin by *Clostridium botulinum* was, in some cases, higher when the organism was grown in extracts of SCP products than in Robertsons meat medium. In some cases a 5 times higher yield was obtained in SCP extract. In suspensions of SCP however toxin production was generally low

These results indicate that pathogenic, and potentially pathogenic bacteria and toxin producing bacteria can grow and produce toxins in SCP substrates, and that SCP may be utilized as substrate for cultivation of bacteria

* The term "Single-cell protein" was introduced in 1966 by professor *C Wilson* Massachusetts Institute of Technology to designate food- and feedstuffs made out of cells of microorganisms such as bacteria, fungi, yeasts and algae. For introductory information see *Mateles R I & Tannenbaum S R* (Eds.) Single-cell protein. The M I T Press, Cambridge (Mass.) 1968, p. 3–7 and *Bhattacharjee J K* Microorganisms as potential sources of food. Advanc. appl. Microbiol *13* 139–161 1970

Acta path. microbiol. scand. Sect. B, 85: 351–354, 1977

SMALL SPHERICAL VIRUSES IN FAECES FROM GASTROENTERITIS PATIENTS

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Kjeldsberg, E. Small spherical viruses in faeces from gastroenteritis patients. Acta path. microbiol. scand. Sect. B, 85: 351–354, 1977.

Faecal samples from 238 patients with gastroenteritis were examined by direct electron microscopy using grids with thin carbon film. Of these samples 18 were found to contain Norwalk agent-like particles, calicivirus, astrovirus and parvovirus-like particles. Immune electron microscopy was performed on serum pair and faeces from one of the patients with astrovirus. An antibody response was demonstrated, suggesting that the virus was the etiological agent of the infection.

Key words: Gastroenteritis; electron microscopy; virus.

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Received 12.iv.77 Accepted 23.v.77

Ever since Kapikian et al. (1972) and Bishop et al. (1973) described virus-like particles as causative agents in acute infantile gastroenteritis, electron microscopy techniques have been widely used to identify structurally characteristic viruses in stools from infants and children. The Norwalk agent (5) was the first small spherical virus to be associated with acute infectious gastroenteritis. Parvovirus-like particles, 20 nm in diameter, were seen both in faeces from persons with gastroenteritis and in faeces from persons not suffering from the disease (3, 9). Virus-like particles, 28 nm in diameter named astrovirus due to the star-shaped configuration of the surface, have also been observed both in stools from babies with gastroenteritis and in stools from normal babies (6, 7). Recently particles with morphology indistinguishable from feline calicivirus (1, 8, 11) were observed in faecal samples from babies with some history of enteritis or "loose stools".

A total of 238 faecal samples from gastroenteritis patients, mainly children, were examined by electron microscopy. The samples were sent to the laboratory from all over Norway during the period August 1976 to February 1977. The purpose was to examine the kind of viruses associated with gastroenteritis in this country. It was thought that the use of thin carbon supports to intensify the image contrast might make it possible to recognize by direct electron microscopy not only the well established rota- and adenoviruses but also the small spherical viruses reported from other countries.

Materials and Methods

Extraction of Faecal Samples

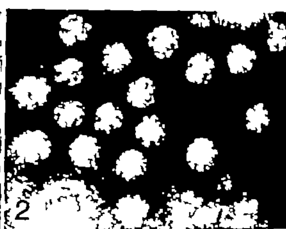
Faeces was suspended to 10–20 per cent (v/v) in Hanks' salt solution supplemented with 0.5 per cent bovine serum albumin and antibiotics. The suspension was shaken for 15 min at room temperature, centrifuged 45 min at 3000 rev/min, and the supernatant was stored at 20 C.

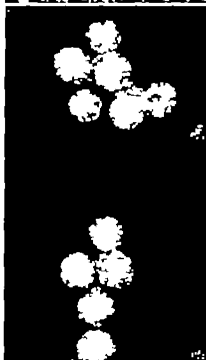
Direct Electron Microscopy

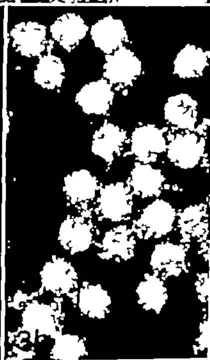
a) *Grids* (200 mesh) were covered with formvar film. Carbon was then evaporated on the formvar as a 6–7 nm thick layer. Afterwards the formvar film was removed with ethylene dichloride according to standard procedures.

b) *Faecal extract* (2.5 ml) was centrifuged 20 min at 5000 rev/min, and the supernatant was recentrifuged 90 min at 19000 rev/min, both in Sorvall RC2-B centrifuge with a SS-34 rotor. The pellet was suspended in 50 µl distilled water.

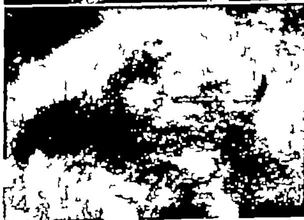



2




3b


3c


4


5
50 nm

After being mixed with an equal volume of 2 per cent phosphotungstic acid (PTA) pH 7 drop was placed on a carbon coated grid. After 30 excess fluid was removed with filter paper and the grid was allowed to dry in the air. The grid was examined in a JEM 100B electron microscope at magnification of 50000 ×

) *Cells* from culture tubes were scraped off the tube with rubber policeman, and the cell-virus suspension was centrifuged for 90 min at 19000 rev/min as described abov The pellet was suspended in a drop of distilled water transferred to metal planchet and freeze-thawed 3 times. The specimen was negatively stained with 2 per cent PTA as described for faecal extracts.

Immune Electron Microscopy

Faecal extract was filtered through 25 mm Millipore filter pore size 1.2 µm and 0.45 µm. A 0.8 ml aliquot of stock filtrate was incubated at room temperature for 60 min with 0.2 ml antiserum diluted 1 10. This preparation was centrifuged 90 min at 19000 rev/min and further proceeded as described for direct electron microscopy. The immune electron microscopy test was performed on the faecal sample and acute and convalescent serum from one of the patients with astrovirus, the only patient from whom we received a serum pair

Result

Of the 238 faecal samples examined, 31 (13 per cent) contained rotavirus and 11 (5 per cent) adenovirus. I 18 samples w were able to demonstrate small spherical irus-like particles which could be classified on the basis of their morphological structure.

Faeces from 4 patients contained particles resembling the Norwalk agent (Fig 1). These were about 30 nm in diameter and were seen to hav capsomere surface structure

The irus in 10 of the samples had morphology as described for astrovirus (7) (Fig. 2). The particles were approximately 30 nm in diameter with an almost circular outline. Most of the particles had a surface configuration in the shape of a 5–6 pointed star Immune electron microscopy was performed using acute and convalescent serum from one of these patients, a 12 year old boy The control with phosphate buffered saline (PBS) showed clumped particles, but no sign of antibody (Fig. 3 a) Aggregates, probably containing antibody between the particles, were seen with acute serum (Fig. 3 b) With convalescent serum (Fig 3 c) the irus particles were heavily coated with antibody and the surface structure completely obscured. The large amount of antibody was probably responsible for the aggregation of the virus particles.

Calicivirus was found in samples from 3 patients. The virus (Fig. 4) was spherical, 30 nm in diameter and with a "star of David"-like configuration. This star-like ppearance is characteristic for the calicivirus group (1 11)

The parvovirus-like particles found in one faecal sample are shown in Fig. 5 These measured 20–22 nm in diameter Empty particles were occasionally observed. No specific surface structure could be seen.

None of these small spherical iruses demonstrated by electron microscopy would grow in cultures of monkey kidney and human amnion cells except for one of the caliciviruses. This gav CPE in one passage and was demonstrated by electron microscopy in two passages of GMK cells.

Discussion

The parvovirus-like particles with no recognizable surface structure might be mistaken for bacteriophages Comparison of the morphology of the irus, however, with earlier published electron micrographs of phage ØX 174 phage MS2, porcine parvovirus and mink enteritis virus (10) indicates human virus rather than bacteriophage.

The antibody response in one of the patients with astrovirus, shown by immune electron microscopy suggested that this irus was the etiological agent of the infection. If the material seen between the virus particles after incubation with acute serum was antibody this antibody may have been acquired during a previous infection with the same irus, or it may have been the result of an early antibody response. None of the other faecal samples were examined by immune electron microscopy and w cannot claim that the small iruses demonstrated in the other cases were the cause of the disease, as lack of paired antisera prevented us from making further investigations.

The presence of rota- and adenovirus in the faecal samples was not surprising as these iruses are wellknown causes of gastroenteritis. Our fa-

Fig 1 Norwalk agent-like particles in faecal extract

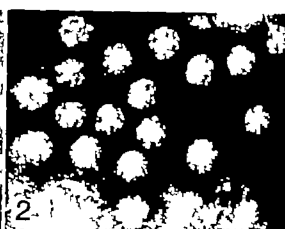
Fig 2 Astrovirus particles in faecal extract.

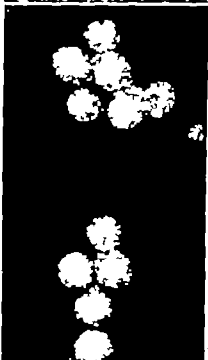
Fig 3 Immune electron microscopy of astrovirus incubated with) PBS b) acute serum,) convalescent serum

Fig 4 Calicivirus in faecal extract.

Fig 5 Parvovirus-like particles in faecal extract. All micrographs are presented at 25,000 × magnification

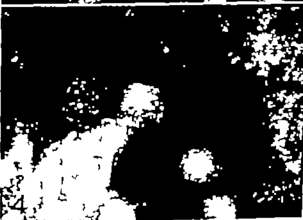



2




3b


3c


4


5
50 nm

A SIMPLE BLOOD AGAR PLATE MEDIUM FOR DEMONSTRATION OF THE STREPTOCOCCAL SERUM OPACITY REACTION

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Belfrage-Sanadn, I. & Christensen, P. A simple blood agar plate medium for the demonstration of the streptococcal serum opacity reaction. Acta path. microbiol. scand. Sect. B, 85 355–356, 1977

Blood agar plates for demonstration of the streptococcal serum opacity reaction were prepared by pouring into Petri dishes four layers, one above the other in the following sequence 1 agar; 2. horse-sheep blood mixed with agar; 3 porcine serum in agarose and 4 agarose. The strains to be tested were streaked with a loop on the plates and a control medium was prepared as described above, but without porcine serum. After incubation for 18 hours at 37° C, the streptococci producing opacity factor showed greenish zones around the colonies or else no haemolysis at all on the plates containing porcine serum, in contrast to a clear beta-haemolysis on the control plates. Opacity reaction negative streptococci showed the same clear beta-haemolysis on both plates. Concordant results were obtained by this method and a standard method for demonstration of opacity factor.

Key words Group A streptococci opacity factor.

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Received 6.ix 77 Accepted 22.ix.77

The test for opacity factor production is useful in streptococcal typing, since approximately one-third of the M-types of group A streptococci constantly form the opacity factor whereas all members of the remaining types fail to do so (2). *Maxted et al.* (2) described two methods, the "tube method" and the "slide method" both based on demonstration of the opacity factor in broth culture supernatant or a cell-extract of the strain to be tested.

The present paper describes a simple blood agar medium on which it was possible to distinguish between streptococci producing opacity factor (SOR+) and opacity reaction negative (SOR−) strains.

Materials and Methods

Strains Fifty-seven group A streptococcal strains were selected at random from among the strains obtained from routine specimens sent to our laboratory. Serological grouping was performed as described previously (1).

Demonstration of the serum opacity factor Opacity factor was demonstrated as described by *Maxted et al.* (the slide method) (2) Overnight culture supernatant from Todd Hewitt broth was used, and in few cases acid extracts were also prepared (2).

The blood agar plates for demonstration of the streptococcal opacity reaction were prepared by pouring four layers, one abov the other into Petri dishes (9 cm diameter, A/S NUNC, Denmark) in the following sequence: 1. 10 ml per plate of 40 g blood agar base (Oxoid, CM 55) in 1 l distilled water; 2. 10 ml horse-sheep blood agar: 7 per cent (vol./vol.) horse blood and 7 per cent sheep blood mixed with agar prepared as described above, except that blood agar base No. 2 (Oxoid CM 271) was used 3 new layer of 4 ml of equal parts of porcine serum and 1 per cent (w./vol.) agarose

ability to demonstrate small spherical viruses earlier may be due to the difficulty of recognizing these among the material usually present in faecal extracts. Suspensions of faeces may contain bacteria, broken flagellae cellular fragments and bacteriophages. By using specimen grids with a 6–7 nm thick carbon support instead of the about ten times thicker formvar carbon coated film the contrast in the virus surface structure is intensified as *fewer inelastically scattered electrons contribute to* the electron image (4) Therefore it should be easier to distinguish the Norwalk agent, the calici virus and the astrovirus from other groups or from non-viral material

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Fig 1 A SOR+ strain (right) and a SOR– strain (left) on the blood agar plate medium containing porcine serum.

(Miles Lab., Ltd., England) in PBS 4 5 ml 1 per cent agarose. The third layer was thus identical with the preparation used in "the slide method" The temperature of the agarose with or without serum was kept during pouring at 50 C after melting

Control plates were prepared by replacing the porcine serum with 0 9 per cent NaCl

The strains to be tested were taken from blood agar plates and streaked directly onto the plates, care being taken not to cut through the upper agarose layer The plates were incubated overnight at 37 C.

Results

On the blood agar plates containing porcine serum the opacity factor positive group A streptococci caused a change in the clear beta haemolytic zone around the colonies seen on the control plates, to either (a) no haemolysis at all, or (b) a green opalescent "haemolysis" Most of the SOR+ strains showed the latter appearance The SOR– strains continued to show the bright beta haemolysis on the blood agar plates containing porcine serum

Provided that the control plates were included, there was no difficulty in differentiating between the SOR+ and SOR– organisms on the blood agar plates. Fig 1 shows SOR+ and a SOR– strain on blood agar containing porcine serum. The 57 strains were tested by both the blood agar plate method and the "slide method" The results obtained by both methods were identical. Two strains proved to be SOR+ by the slide method only when acid extracts were used, but presented no difficulty in interpretation on the blood agar plates. In all 30 strains were SOR+ and 27 SOR

Discussion

The method described in the present investigation offered two main advantages. Firstly the technique allowed differentiation of SOR+ and SOR colonies by differential streaking This might prove useful not only in order to facilitate detection of double infection in a patient, but also when studying the genetics of the opacity factor Secondly the method was more simple than the others described in that it allowed direct streaking and interpretation without cultivation on broth. *Widdowson et al* (4) have also described a plate agar method in which opacity factor was demonstrated as a halo around deep colonies growing in 50 per cent horse serum agar However we have found that the technique described in the present report facilitates interpretation considerably (*Belfrage-Sandén & Christensen* unpublished observation)

No attempts were made in the present investigation to elucidate the reason for the impaired haemolysis of SOR+ strains on the plates as described Poor haemolysis of SOR+ streptococci was also noted by *Pinney* (3) who cultured streptococci on horse blood agar plates. *Pinney* (3) suggested that the opacity factor might interfere with the streptolysin S binding to alpha-lipoprotein, or that SOR+ strains might produce less streptolysin S than SOR– strains. However we found no impairment of haemolysis on the plates not containing porcine serum.

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Acta path. microbiol scand. Sect. B, 85 357–362 1977

TYPE CLASSIFICATION OF GROUP B STREPTOCOCCI BY THE FLUORESCENT ANTIBODY TEST

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Bevanger L. & Mæland, J. A. Type classification of group B streptococci by the fluorescent antibody test. Acta path microbiol scand. Sect. B, 85 357–362, 1977

Fluorescein-isothiocyanate conjugates were prepared from rabbit anti-type Ia, Ib, II and III sera, and used to classify 90 strains of group B streptococci isolated from the genital tract of puerperal women. Of the 84 strains that were typeable, the numbers belonging to serotypes Ia, Ib, Ic, II and III were 7 14 6 37 and 20 respectively The anti-type Ib conjugate showed cross-reactivity with 21 of the type II strains and one of the type III strains. Absorption of the anti-type Ib conjugate with bacteria of a type Ic strain and testing of enzyme-digested streptococci indicated cross-reactivity due to antibody to the Ibc protein antigen of group B streptococci The potential usefulness of this antigen in the classification of type II and type III strains is discussed

Key words Group B streptococci type classification fluorescent antibody test

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Received 16 77 Accepted 4. ii.77

Group B streptococci were originally classified serologically by *Lancefield* (13) on the basis of specific carbohydrate (CHO) antigens, called by her the type I, II and III antigens. Subsequently type I strains were separated into types Ia and Ib (14) and finally Ic was recognized as a third serotype of the type I complex (23 26) Type Ic strains possess a CHO antigen closely related to or identical with that of type Ia strains, and a protein antigenic fraction named the Ibc protein (15 23 24). The Ibc protein is present also in type Ib strains (23 24) and has been detected in some type II strains (15 24 25) Table 1 summarizes some of the present knowledge of antigens characterizing group B streptococci of the type I complex.

Group B streptococci are frequently isolated from various sites of the human body (24) Among diseases caused by this bacterium, neonatal septicaemia and meningitis are particularly dangerous for the patient (3 4 7 10) For the classification of group B strains, investigators have used mostly immunoprecipitation tests (9 13 14 16 17 24) in preference to the fluorescent antibody (FA) test (12, 18)

A total of 90 strains of group B streptococci were isolated in our laboratory from the genital tract of puerperal women (5) The bacteria have been further characterized by determination of serotypes by means of the FA test.

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els, diameter 3 mm, were made with 7 mm between the adjacent margins. The wells were filled [with] undiluted FITC conjugates or HCl extract of streptococci containing the CHO antigens (13). The extracts were prepared by treatment of the bacteria with 0.2 N HCl at 50° C for 2 h, after which the mixture was centrifugated and the supernatant neutralized with NaOH (17). The slides were incubated in wet chamber at 20° C for 20 h then at 4 C, and readings were made daily for up to four days.

RESULTS

FITC-conjugated globulins were prepared from several rabbit antisera to each of the type strains of group B streptococci and tested for FA activity against homologous and heterologous bacteria. One conjugate against each of the type strains Ia, Ib, II and III was selected for serotyping. When examined unabsorbed, these conjugates showed strong FA staining of the homologous strains and weak or no staining of heterologous strains. After cross-absorption, FA activity was observed against the homologous bacteria only. In the agar gel diffusion test, undiluted samples of the cross-absorbed conjugates showed precipitation with the HCl extract of the homologous type strain, but not with extract from any of the heterologous strains. The strains used for cross-absorption and some characteristics of the FITC conjugates are shown in Table 2. Dilutions corresponding to the titres shown were used for type classification of the group B streptococci.

TABLE 2. *Serotype of Bacteria Used for Cross-Absorption and Data Characterizing the FITC Conjugates Against Type Strains of Group B Streptococci*

FITC conjugate against type	Absorption with type	Protein (g/l)	F/P ratio mol/mol	Titre [1])
I	II	5.8	5.8	16
Ib	I	8.4	3.2	32
II	III	8.2	3.1	16
III	I	10.0	4.0	16

[1]) Reciprocal of highest dilution of conjugate showing 4+ reaction against homologous bacteria.

TABLE 3. *A total of 90 Strains of Group B Streptococci Listed According to Staining Intensity (— to 4+) with FITC Conjugates*

No. of strains	FITC Conjugate against type			
	Ia	Ib	II	III
7	4+	—	—	—
14	—	4+	—	—
16	—	—	4+	—
19	—	—	—	4+
6	3—4+	2—3+	—	—
21	—	2—4+	4+	—
1	—	4+	—	4+
6	—	—	—	—

The results of FA testing of 90 group B isolates are shown in Table 3. A total of 84 strains showed FA staining by one or two of the conjugates and were considered typeable whereas six strains showed no staining even when higher concentrations of the conjugates were used. These strains were considered non-typeable. FA staining by one conjugate only was observed with 56 strains. The remaining 28 isolates showed FA staining by two of the conjugates, all of them by the anti-type Ib conjugate and, in addition, the anti type Ia, II or III conjugate.

Bacteria of each of the 28 strains were digested with trypsin and examined by the FA test as before. Fluorescence intensity was diminished with some of the treated strains, but all of them still showed staining by the anti-type Ib conjugate. However after digestion of the bacteria with pepsin, FA staining by the anti-type Ib conjugate was no longer demonstrable. The pepsin-digested bacteria still exhibited FA staining by the anti-type Ia, II or III conjugate as before (Table 3). A portion of the anti-type Ib conjugate was absorbed with the prototype Ic strain A909 then diluted 1 to 16, and tested against bacteria that showed cross-reactivity with the unabsorbed conjugate. The absorbed sample showed no FA activity against bacteria of any of these strains. However the absorbed sample stained type Ib strains in the same way as before the absorption.

The results of the type classification per

TABLE 1 *Carbohydrate (CHO) and Protein Antigens Characterizing Group B Streptococci of the Type I Complex (15)*

Serotype	Type-specific CHO antigen	Cross-reactive CHO antigen	Protein antigen
Ia	Ia	Iabc	None
Ib	Ib	Iabc	Ibc
Ic	Ia	Iabc	Ibc

MATERIAL AND METHODS

Strains

Type strains of group B streptococci were received through the courtesy of *Rangdi Holth Haug*, Oslo The following type strains were used Type Ia, ATCC 12400 *Lancefield* 090 type Ib, ATCC 12401 *Lancefield* H36B type Ic, A909 type II ATCC 12973 *Lancefield* V8 type III ATCC 12403 *Lancefield* D136C type R, NCTC 9828 type X, NCTC 9829

The clinical isolates examined were identified as group B streptococci by a ring precipitation test (5) All strains were kept at —80 C in Stuart transport medium and thawed immediately before culturing

Culture

Streptococci used for immunization of rabbits or for FA testing were cultured on the fermentation medium described by *Watkins & Flynn* (20) supplemented with one per cent glucose No indicator was added. Bacteria were cultured at 37 C for 20 h and harvested in phosphate-buffered saline, pH 7 2 (PBS) Streptococci cultured in Todd Hewitt broth at 37 C for 20 h were used for absorption of the fluorescein-conjugated globulins or preparation of HCl extracts The microorganisms were collected by centrifugation at 8000 × *g* for 10 min and washed once with PBS

Immunization

Streptococci of the various types were suspended in PBS containing 0.3 per cent formalin and adjusted to a density giving an optimal transmission of 60 per cent at 540 nm. Bacteria of the type III strain were digested with pepsin (Calbiochem) for 2 h as reported previously (11) in order to destroy the R protein carried by this strain (?2 18) Bacteria of the other serotypes were used untreated. Rabbits were injected intravenously on five successive days each week during a period of three weeks, with doses increasing from 0 5 to 1 ml of the bacterial suspension. The animals were bled by heart puncture six days after the last injection, and the sera were stored at —80 C.

Preparations of Conjugates

Precipitation of rabbit antisera with ammonium sulphate and conjugation of the immunoglobulin fraction with fluorescein-isothiocyanate (FITC BBL, Cockeysville) were performed essentially as described by *Thomason et al* (19) Ammonium sulphate was eliminated before conjugation and free FITC after conjugation by gel filtration of the protein solution on a Sephadex G 25 (Pharmacia) column. For the conjugation, 0 03 mg of FITC was used per one mg of protein, assayed by the biuret method (8) FITC, protein concentration and molar FITC to protein ratio (F/P) of the conjugates were determined spectrophotometrically (21) Twofold dilutions of the conjugates were examined against homologous and heterologous type strains of the streptococci (see below) Cross absorption of the conjugates was then performed to eliminate FA staining of the heterologous strains. One volume of undiluted conjugate was mixed with one-half volume of washed and packed bacteria, incubated at ?0 C for 30 min then at 4 C for 20 h, and centrifuged at 8000×*g* for 10 min The supernatant was examined by the FA and agar precipitation tests. FITC-conjugated globulins from the serum of non-immunized rabbits were prepared in the same way Conjugates ready for use were stored in small portions at —80 C and thawed once.

Fluorescent Antibody (FA) Test

Smears were prepared from streptococci suspended in PBS dried in the air and heat-fixed gently The smear was covered with the FITC conjugate incubated in a wet chamber at 20 C for 30 min, and washed for 15 min in three different baths of PBS The smear was mounted under a cover slip with phosphate-buffered glycerol, pH 7.2 and read under a Leitz-Ortholux fluorescence microscope (400 ×) equipped with incident light, Osram HBO 200 mercury lamp KP490, KP540 and K480 exciter filters, K515 barrier filter and TK510 chromatic reflector In some experiments, streptococci were treated with pepsin (Calbiochem) for 2 h or with trypsin (Calbiochem) for 3 h a reported previously (11) and then exam ned by the FA test. Controls included smears of bacteria not incubated with FITC conjugates and smears incubated with conjugates from non-immunized rabbits The fluorescence intensity was recorded as to 4 + (19)

Agar Gel Diffusion

Double-diffusion slides were prepared, using one per cent agar (Noble, Difco) in PBS Circular

ells, diameter 3 mm, were made with 7 mm between the adjacent margins. The wells were filled with undiluted FITC conjugates or HCl extract of streptococci containing the CHO antigens (13). The extracts were prepared by treatment of the bacteria with 0.2 N HCl at 50° C for 2 h, after which the mixture was centrifugated and the supernatant neutralized with NaOH (17). The slides were incubated in a wet chamber at 20 C for 20 h then at 4 C, and readings were made daily for up to four days.

RESULTS

FITC-conjugated globulins were prepared from several rabbit antisera to each of the type strains of group B streptococci and tested for FA activity against homologous and heterologous bacteria. One conjugate against each of the type strains Ia, Ib II and III was selected for serotyping. When examined unabsorbed, these conjugates showed strong FA staining of the homologous strains and weak or no staining of heterologous strains. After cross-absorption, FA activity was observed against the homologous bacteria only. In the agar gel diffusion test, undiluted samples of the cross-absorbed conjugates showed precipitation with the HCl extract of the homologous type strain, but not with extract from any of the heterologous strains. The strains used for cross-absorption and some characteristics of the FITC conjugates are shown in Table 2. Dilutions corresponding to the titres shown were used for type classification of the group B streptococci.

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Ia	II	5.8	5.8	16
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II	III	8.2	3.1	16
III	I	10.0	4.0	16

[1] Reciprocal of highest dilution of conjugate showing 4+ reaction against homologous bacteria.

TABLE 3. *A total of 90 Strains of Group B Streptococci Listed According to Staining intensity (— to 4+) with FITC Conjugates*

No. of strains	FITC Conjugate against type Ia	Ib	II	III
7	4+	—	—	—
14	—	4+	—	—
16	—	—	4+	—
19	—	—	—	4+
6	3—4+	2—3+	—	—
21	—	2—4+	4+	—
1	—	4+	—	4+
6	—	—	—	—

The results of FA testing of 90 group B isolates are shown in Table 3. A total of 84 strains showed FA staining by one or two of the conjugates and were considered typeable whereas six strains showed no staining even when higher concentrations of the conjugates were used. These strains were considered non-typeable. FA staining by one conjugate only was observed with 56 strains. The remaining 28 isolates showed FA staining by two of the conjugates, all of them by the anti-type Ib conjugate and, in addition, the anti-type Ia, II or III conjugate.

Bacteria of each of the 28 strains were digested with trypsin and examined by the FA test as before. Fluorescence intensity was diminished with some of the treated strains, but all of them still showed staining by the anti-type Ib conjugate. However after digestion of the bacteria with pepsin, FA staining by the anti-type Ib conjugate was no longer demonstrable. The pepsin-digested bacteria still exhibited FA staining by the anti-type Ia, II or III conjugate as before (Table 3). A portion of the anti-type Ib conjugate was absorbed with the prototype Ic strain A909 then diluted 1 to 16, and tested against bacteria that showed cross-reactivity with the unabsorbed conjugate. The absorbed sample showed no FA activity against bacteria of any of these strains. However the absorbed sample stained type Ib strains in the same way as before the absorption.

The results of the type classification per

formed are summarized in Table 4 Typeable strains, which comprised 84 of 90 isolates could be assigned to one or other of the serotypes Ia Ib II or III the majority to types II and III Strains belonging to any of the CHO antigen types Ia, II and III could be further subdivided on the basis of cross-reactivity with the Ib conjugate.

TABLE 4 *A Total of 84 Strains of Group B Streptococci Listed According to Carbohydrate Antigen Types*

FA staining with	No. of strains of antigen type			
	Ia	Ib	II	III
One FITC conjugate only	7	14	16	19
Two FITC conjugates a)	6b)	0	21	1

a) The anti-type Ib conjugate in addition to the anti-type Ia II or III conjugate
b) Regarded as strains of serotype Ic

DISCUSSION

When prepared from the serum of rabbits immunized with type strains of group B streptococci FITC-conjugated antibody globulins could be used to classify clinical group B isolates. However type specificity of the conjugates, as indicated by the results of FA and agar precipitation testing required absorption with bacteria of a heterologous serotype

FA testing using a conjugate against bacteria of each of the serotypes Ia Ib II and III permitted type classification of 84 out of 90 group B strains examined The non typeable isolates may belong to serotypes X or R, but this remains to be determined FA staining by one of the conjugates only was observed with 56 strains Accordingly these strains belonged to one or other of the CHO antigen types. Similarly all the remaining 28 strains were assigned to one or other of the CHO antigen types Ia, II or III However without exception, these latter strains also showed FA staining by the anti type Ib conjugate This cross-reactivity was eliminated when the bacteria were digested with pepsin before testing but not with trypsin and by absorption of the Ib conjugate with bacteria of a type Ic strain. The results accord with cross-reactivity due to antibodies to antigen of protein nature. FA staining by the anti type Ib conjugate of the prototype X or R strain was not observed, thereby minimizing the possibility that the X or R protein antigen was responsible. However the cross-reactivity observed can be explained on the basis of antibody to antigens of the Ibc protein fraction, supported by the following observations The results reported indicate production of the Ibc protein antigen by all type Ib strains (23) concordant with antibody to this antigen in the Ib conjugate used in this study the Ibc antigen is susceptible to pepsin but only partially susceptible to trypsin digestion (23) the protein antigen is also produced by the type Ic strain A909 (23) used in this study to absorb the cross-reacting antibodies from the anti type Ib conjugate.

On the assumption that the cross-reactivity of the Ib conjugate was mediated by antibodies to the Ibc protein antigen it may be concluded that out of 13 strains which carried the CHO antigen Ia, six belonged to serotype Ic This is similar to the proportionate number of Ia and Ic strains among group B isolates demonstrated by others (1 2, 24) The Ibc protein was detected in 21 out of 37 type II strains, a frequency exceeding that recorded by others on the basis of precipitation tests (1 24) That type III strains may produce the Ibc protein antigen has been suggested (25) and was further substantiated in this study The results obtained suggest that the Ibc protein antigen may form a basis for subdivision of strains of the CHO antigen type II or III not only those of type Ia.

In the Ibc protein fraction from the type Ic strain A909 two precipitinogens were demonstrated (23) The potential usefulness of this antigen fraction in the classification of group B streptococci merits further study

The streptococci examined in this study were isolated from the genital tract of puerperal women The distribution of the various antigen types in this collection of strains was essentially similar to that of genital group B

strains isolated by others (1 2, 6, 24) The majority of the strains studied by us either belonged to type II or type III 20 out of 84 strains to type III. Neonatal infections by group B streptococci are usually caused by type III strains (1 2, 24) The FA test has the advantage of rapid identification of the serotype of the bacteria when preventive measures are to be taken with genital carriers of group B streptococci.

The technical work made by Mrs *Augusta Nass* is greatly appreciated.

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Acta path. microbiol. scand. Sect. B, 85: 365–373, 1977

THE ULTRASTRUCTURE AND DISTRIBUTION OF MICROPORES IN THE VARIOUS DEVELOPMENTAL FORMS OF *EIMERIA BRUNETTI*

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Ferguson, D. J. P., Birch-Andersen, A., Hutchison, W. M. & Siim, J. Chr. The ultrastructure and distribution of micropores in the various developmental forms of *Eimeria brunetti*. Acta path. microbiol. scand. Sect. B, 85: 365–373, 1977.

The structure and distribution of micropores in the various developmental stages of *Eimeria brunetti* was examined. Micropores were observed in all the endogenous forms with the exception of the microgamete. Oocysts from chicken faeces were also examined at various stages of sporulation and micropores were demonstrated in zygotes, sporoblasts, sporozoites, and the residual cytoplasmic masses. The number of micropores per organism appeared to be correlated with the surface area of the organisms irrespective of whether these were endogenous or sporulating forms. The increase in the number of micropores did not appear to be related to micropore activity because seemingly active micropores were observed only in the trophozoites, in the early multinucleate forms (early schizonts and microgamonts) and in the early macrogamonts. All these forms, however, possessed relatively few micropores. No active micropores were ever observed within the sporulating oocysts.

Key words: *Eimeria brunetti*; micropores; endogenous forms; oocysts; chickens.

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Received 8. ii. 77. Accepted 8. iii. 77

The micropore was first observed in the sporozoite of *Plasmodium falciparum* (12) and has subsequently been reported as a characteristic organelle of the *Sporozoa*. Detailed reviews on the occurrence of micropores within the *Sporozoa* have been published by *Scholtyseck & Mehlhorn* (27) and *Senaud et al.* (29). Within the genus *Eimeria*, the presence of a micropore was first reported in the merozoites of *Eimeria intestinalis* by *Cheissin & Snigirevskaya* (5) and they suggested that it possibly functioned as an "ultracytostome". Within the haemosporidians there is a great deal of evidence for the micropore acting as a cytostome and participating in the ingestion of host material (1, 2). It has been more difficult to find support

Work initiated while a W. Browne Trust Travelling Research Fellow and completed as a Danish Medical Research Council Fellow.

for the ultracytostome hypothesis among the non haemosporidian members of the *Sporozoa*, but the available evidence has recently been reviewed by *Senaud et al* (29)

In all the species of the genus *Eimeria* which have been studied micropores have been observed in the sporozoites and in all the endogenous forms, with the exception of the *microgametes* *Until recently the ultra* structural details of the stages within the oocyst were unknown but in a previous brief communication (11) we have noted the presence of a micropore in the late sporoblast of *Eimeria brunetti* In the present study for the first time all the stages in the complete *life cycle of E brunetti* are examined for the presence of micropores The relative distribu*tion and the apparent functional status in the* various developmental forms will be discussed.

MATERIALS AND METHODS

Examination of the endogenous forms Small intestines of young domestic fowls infected with *E. brunetti* were examined. The methods were similar to those previously described (7) but can be summarized as follows Pieces of the small intestine were fixed in glutaraldehyde and osmium tetroxide and embedded in Vestopal W Thin sections were examined in the electron microscope *after staining with magnesium uranyl* acetate and lead citrate.

Examination of oocysts The oocysts were obtained from chicken faeces and were allowed to sporulate for various time intervals before being treated by the double sectioning technique described previously (4) This involved pre-embedding of oocysts in crossed-linked bovine serum albumin and deep freezing in liquid nitrogen prior to cryo-sectioning which was followed by primary fixation. Further treatments for ultramicrotomy and electron microscopy were as summarized above and the observations were based on the examination of approximately 4000 electron *micrographs.*

Figures 1–15 are all micrographs which illustrate the presence of micropores in the various stages of the endogenous development of *E brunetti* within the epithelial cells of the small intestines of infected chickens.

Figures 16–23 are all *micrographs* which illustrate the presence of micropores in the various stages of the sporulation of oocysts of *E. brunetti* *in the external environment.*

A double bar (=) on a micrograph represents 1 µm and a single bar (—) represents 100 nm.

The *following abbreviations* are *used* through out AL = amorphous layer B = basal body C = collar of dense material DB = dense body (anlage of refractile body) ER = rough endoplasmic reticulum EV = electron translucent vacuole FL = flagellum G = Golgi body LM = limiting membrane M = microneme ME = merozoite MI = mitochondrion MP = micropore MV = multimembraneous vacuole N = nucleus O = outer layer of the oocyst wall OW = oocyst wall PG = polysaccharide granule PL = pellicle PV = *parasitophorous* vacuole R = ribosome RH = rhoptry RM = residual cytoplasmic mass SH = schizont SP = sporozoite WFB I = wall forming bodies of type I WFB II = wall forming bodies of type II

Fig 1 A section through part of a multinucleate stage showing two nuclei and an active micropore. 30 000 ×

Fig 2 An enlargement of the active micropore in Fig 1 showing that it consists of an invagination of the limiting membrane with a collar of dense material round the neck (arrows) 90,000 ×

Fig 3 Part of a *fully formed merozoite showing* the typical anterior organelles (rhoptries and micronemes) Note the inactive micropore situated just anterior to the nucleus. *30 000* ×

Fig 4 A section through an inactive micropore situated on *the pellicle* of a merozoite The micropore is formed by an invagination of the outer membrane of the pellicle and the collar (arrows) *by an invagination of the inner layer* of the pellicle. 90 000 ×

Fig 5 A section through part of a schizont showing a merozoite as it is formed by a protrusion from the surface of the schizont. Note the inactive micropore on the limiting membrane of the schizont. 45 000 ×

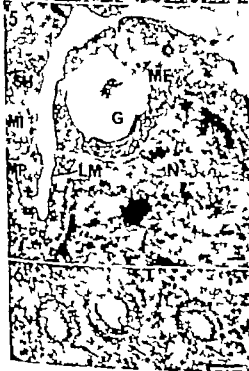
Fig 6 A section through the periphery of a microgamont. A *group of three cross-sectioned* micropores is present (arrows) 90 000 ×

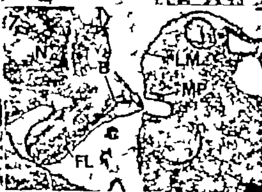
Fig 7 Part of an early microgamont showing the basal body and flagellum of a developing microgamete An inactive micropore is present on the *limiting membrane of the* cytoplasmic mass of the organism 45 000 ×

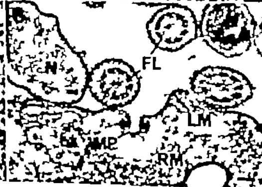
Fig 8 A section through a mature microgamont showing the nuclei and flagella of some of the microgametes. Note the inactive micropore on the limiting membrane of the residual cytoplasmic mass of the microgamont. 45 000 ×


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In all the species of the genus *Eimeria* which have been studied, micropores have been observed in the sporozoites and in all the endogenous forms, with the exception of the microgametes Until recently the ultra structural details of the stages within the oocyst were unknown but in a previous brief communication (11) we have noted the presence of a micropore in the late sporoblast of *Eimeria brunetti*. In the present study for the first time all the stages in the complete life cycle of *E brunetti* are examined for the presence of micropores The relative distribution and the apparent functional status in the various developmental forms will be discussed.

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Fig 6 A section through the periphery of a microgamont A group of three cross-sectioned micropores is present (arrows) 90 000 ×

Fig 7 Part of an early microgamont showing the basal body and flagellum of a developing microgamete An inactive micropore is present on the limiting membrane of the cytoplasmic mass of the organism. 45 000 ×

Fig 8 A section through a mature microgamont showing the nuclei and flagella of some of the microgametes. Note the inactive micropore on the limiting membrane of the residual cytoplasmic mass of the microgamont. 45,000 ×

RESULTS

Ultrastructure. The micropore was found to consist of a circular invagination (70–80 nm in diameter) of the limiting membrane of the organism (Figs 6, 13 and 18). In forms which possessed a pellicle the inner layer of the pellicle was also seen to invaginate for a short distance (60–70 nm) (Figs 3 and 4) and thus a dense collar (120–150 nm in diameter) was present round the neck of the micropore (Figs 6, 13 and 18). This collar was also present in organisms which were limited only by a single unit membrane (Figs 2, 10 12, 14 and 17). Based on the depth of the invagination of the limiting membrane, the micropores could be divided into two types, probably of different functional significance. The first consisted of seemingly inactive micropores in which the invagination was only 90–140 nm deep (Figs 4 5 7 8 12, 14 17 20 21 and 22). The second consisted of the seemingly active micropores in which the invagination was much deeper (370–420 nm) and which in addition, presented a bulbous appearance (Figs 1 2 9 and 10). The structural characteristics of the active and inactive micropores are diagrammatically presented in Text Fig 1

Distribution

The endogenous forms. Active micropores were observed only in the trophozoites, in the early multinucleate stages (Fig 1) which develop into schizonts or microgamonts, and in the early macrogamont (Figs 9 and 10). The number of active micropores observed in these stages was low. If present, normally only one was observed per section, but occasionally two could be found in a single section.

In the later developmental stages only inactive micropores were observed. This applied to organisms which had developed to the stage where schizonts could be differentiated from microgamonts because of the initiation of merozoite or microgamete formation and also to organisms throughout the later stages of schizogony and microgametogenesis (Figs 5 6 and 7). In the schizonts, the number of micropores was low although a somewhat higher number was observed in the large 1st generation schizonts. In the microgamont, which possessed deep invaginations of the limiting membrane of the organism, the number of micropores appeared relatively higher. In this stage the micropores were often observed in groups with as many as three present in an area of 0.15 µm (Fig 6)

In the merozoite formed by schizogony only a single micropore was observed per organism. This micropore was of the inactive type and was situated on the pellicle just anterior to the nucleus (Figs 3 and 4)

No micropores were observed in the microgametes.

Fig 9. Part of an early macrogamont showing the nucleus, some multimembraneous vacuoles, some developing WFB I and Golgi body. The organism is limited by a single membrane on which an active micropore is present. 30,000 ×

Fig. 10 An enlargement of the active micropore in Fig 9. Note that the micropore consists of an invagination of the limiting membrane with col-lar of dense material round the neck region (arrow) 90,000 ×

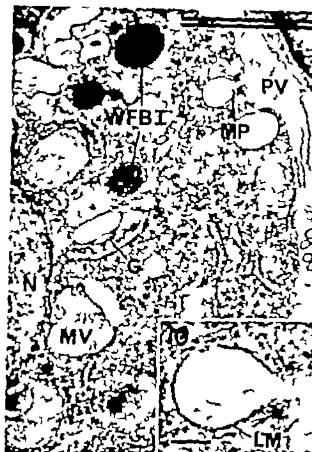
Fig 11 Part of an oblique section through late macrogamont. In addition to the three cross sectioned micropores (arrow) some WFB I and some WFB II are shown together with number of polysaccharide granules. 30,000 ×

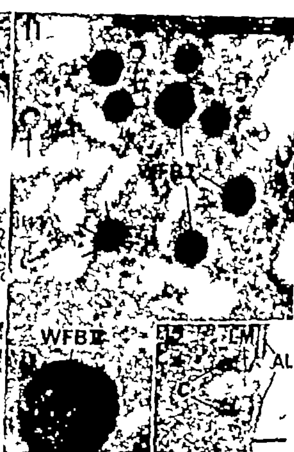
Fig 12 Part of section of macrogamete on hich the amorphous layer exterior to the limiting membrane is well illustrated. An inactive micropore is also present 90,000 ×

Fig 13 A cross sectioned micropore present in an oblique section through the periphery of an organism in hich oocyst wall formation is occurring 90,000 ×

Fig 14 Part of section of forming oocyst. Note the presence of the inactive micropore on the limiting membrane of the cytoplasmic mass below the outer layer of the oocyst wall. 90,000

Fig 15 Part of section through an organism in hich the outer layer of the oocyst wall is present. Note the fiv micropores (arrow) at the periphery of the cytoplasm mass below the outer oocyst all 15,000


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Fig 13 A cross sectioned micropore present in an oblique section through the periphery of an organism in which oocyst wall formation is occurring 90,000 ×

Fig 14 Part of section of forming oocyst. Note the presence of the inactive micropore on the limiting membrane of the cytoplasmic mass below the outer layer of the oocyst wall 90,000 ×

Fig 15 Part of section through an organism in which the outer layer of the oocyst wall is present Note the in micropores (arrow) at the periphery of the cytoplasmic mass below the outer oocyst wall 15,000 ×

Distribution

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In the later developmental stages only inactive micropores were observed. This applied to organisms which had developed to the stage where schizonts could be differentiated from microgamonts because of the initiation of merozoite or microgamete formation and also to organisms throughout the later stages of schizogony and microgametogenesis (Figs 5 6 and 7) In the schizonts, the number of micropores was low although a somewhat higher number was observed in the large 1st generation schizonts. In the macrogamont, which possessed deep invaginations of the limiting membrane of the organism, the number of micropores appeared relatively higher In this stage the micropores were often observed in groups with as many as three present in an area of 0 15 μm (Fig. 6)

In the merozoite formed by schizogony only a single micropore was observed per organism. This micropore was of the inactive type and was situated on the pellicle just anterior to the nucleus (Figs 3 and 4)

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In the merozoite formed by schizogony only a single micropore was observed per organism. This micropore was of the inactive type and was situated on the pellicle just anterior to the nucleus (Figs 3 and 4)

No micropores were observed in the microgametes.

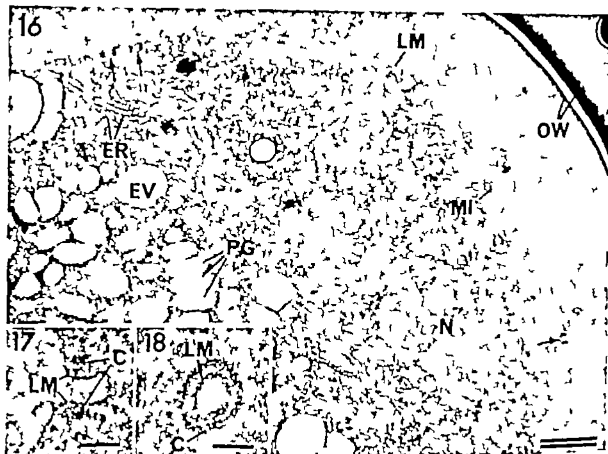


Fig 16 A section through part of a zygote within an oocyst. The cytoplasm contains a nucleus, some polysaccharide granules, some electron translucent vacuoles, some mitochondria, and some rough endoplasmic reticulum. Note the three inactive micropores (arrows) on the limiting membrane of the zygote 15 000 ×

Fig 17 A field of view which shows part of the limiting membrane of a zygote Note the presence of an inactive micropore. 90 000 ×

Fig 18 A section through the periphery of a zygote. Note the presence of a cross sectioned micropore 90 000 ×

In the later developmental stages of the macrogamont and in the fully formed macrogamete the number of micropores was relatively higher (Fig 11) Here as many as six micropores have been observed in two adjacent sections of one organism. After the initial developmental stages of the macrogamont these micropores were all of the inactive type (Fig 12)

In organisms in which the oocyst wall had started to form it was possible to find large numbers of inactive micropores (Figs 13 14 and 15) In a single thin section as many as six were observed situated on the limiting membrane of the cytoplasmic mass below the developing oocyst wall.

Oocysts In the initial cytoplasmic mass, the zygote it was possible to find inactive micropores (Figs 17 and 18) In this case as many as three have been observed in a single thin section (Fig 16)

Inactive micropores were also observed in the early sporoblasts which are formed by the division of the zygote The late sporoblast was found to be limited by two unit membranes and inactive micropores were observed associated with the inner of these membranes (Fig 20) *i.e* with the one which





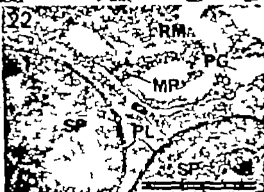




Fig 19 Part of a section through late sporoblast with two inacti micropores (arrow) at the periphery of the organism The cytoplasm contains dense bodies, lectron translucent acuoles, and some rough endop asm reticulum 30,000 ×

Fig 20 A detail of an inactiv micropore from late sporoblast Note that t is the inner of the tw limiting membranes which invaginates to form th micropore. The collar of dense material is present in the neck region (arrow) 90,000 ×

Fig 21 A field of iew showing part of a developing sporocyst in which a sporozoite is being formed. Note the inactiv micropore on the limiting membrane of the cytoplasmic mass from which the sporozoite is being formed 30,000 ×

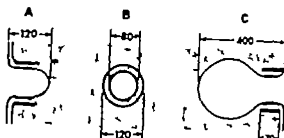
Fig 22 A section through mature sporocyst in which parts of the tw sporozoites can be seen. An inactiv micropore is present on the limiting membrane of the residual cytoplasm mass. 30,000 ×

Fig 23 A section through part of mature sporocyst in which an inactive micropore is present on the pellicle of one of the sporozoites. 30,000 ×

constitutes the plasmalemma and not the outer membrane which is related to the formation of the sporocyst wall Two micropores have been observed in a single thin section through a sporoblast (Fig 19)

In the sporocyst, inactive micropores were also observed on the limiting membrane of the cytoplasmic mass as sporozoite formation was occurring (Fig 21) In addition, micropores could be observed on the residual mass, which was left after completion of sporozoite formation (Fig 22)

The sporozoites formed within the sporocyst each possessed an inactive micropore situated just anterior to their nuclei (Fig 23)



Text Fig 1 A diagrammatical representation of longitudinally sectioned inactive (A) and active (C) micropores. The cross sectional appearance through the neck region is shown in (B) The average dimensions are given in nm.

DISCUSSION

In our previous studies on the endogenous development of *E brunetti* we have referred to the presence of micropores without relating this to their functional status (7 8 9 10) In this study we have made the assumption based on the evidence reviewed by *Senaud et al* (29) that the micropore is functioning as a cytostome giving rise to food vacuoles and that this activity can be related to its structural appearance. We have used the terms active and inactive to describe functional and non functional micropores respectively This is similar to the terminology used by *Michael* (18) and a diagrammatical representation of the two types of micropores is given in our Text Fig 1

The basic ultrastructure of the micropore of *E brunetti* is similar to that reported for the *Eimeria* spp and other members of the *Sporozoa* (for references see 27 29 and 35)

The exact number of micropores in the various developmental stages cannot be ascertained unless complete serial sections through individual organisms can be examined this was not carried out We have examined the relative number of micropores, i e the frequency with which one or more micropores were observed within the sections that were available through the different stages in the life cycle of the organism It was found that the micropores were less frequently observed in the small stages (i.e merozoite, sporozoite and trophozoite) and that the relative number appeared to increase with increased size of the developmental stage examined. The increase in number of micropores per organism was apparently related to the surface area of the organism so that the largest numbers were observed in organisms with the largest surface area (i.e 1st generation schizonts, microgamonts and macrogamonts) Similar observations have also been reported for the endogenous forms of other *Eimeria* spp (13 16 27 31 33) It would appear that formation of the micropore is somehow related to the synthesis of the limiting membrane of the organism. Hypothetically the genome for the synthesis of the plasmalemma could be related to the genome for micropore formation in such a manner that when the surface area of the organism increases the number of micropores would also increase. This would explain the presence of micropores under the forming oocyst wall which has been reported in the present study and also for other *Eimeria* spp. (6 15 35) In this situation it is difficult to attribute a functional significance to the micropore. In addition, the absence of micropores in the microgamete could be due to the very small size of this form. Because microgametes have a very small surface area, formation of their plasmalemma is limited and probably insufficient to trigger off any micropore formation This is in accordance with reported observations in which micropores

are absent in members of the *Sporozoa* which produce small microgametes (see review by *Scholtyseck et al.* (28)) but have been reported to be present in the large microgametes produced by other members such as *Coelotropha durchoni* (20) and *Aggregata eberthi* (24).

In *E. brunetti* active micropores are limited to the early developmental stages of the endogenous forms. These early stages are the actively growing stages and apparently active micropores have only been observed during this growth phase. In the later stages when the organisms are undergoing differentiation (formation of merozoites, microgametes or macrogametes) although the micropores are relatively more abundant, they are all of the inactive type. It would thus appear that in *E. brunetti* the endogenous forms do not feed continually but satisfy their macromolecular requirement during the growth phase. The presence of active micropores in the early stages only corresponds well to their presence during macrogametogenesis in *E. intestinalis* as earlier reported (32). In a number of studies providing evidence for the cytostome function of the micropore in *Eimeria* spp. the micropores were reported to be present in the early developmental stages (14, 26, 29, 34). For *E. acervulina* (18) and *E. magna* (33) however the presence of both active and inactive micropores has been reported in the later stages of macrogametogenesis. It is possible that the differences noted are related to variations between eimerian species. In *E. brunetti* as observed in this study an increase in the number of micropores does not appear to be related to increased nutritional requirements but more to an increase in the surface area of the organism.

Micropores were also found in the stages within the oocyst. This was not completely unexpected since one example of micropores on a developing sporoblastoid of *Plasmodium* micae has been described (30) although micropores have never been observed in the oocysts of the haemosporidia (3 and Garnham personal communication). In addition other parasites belonging to families and genera related to the *Eimeria* have been reported to possess micropores during their sporulation (17, 19, 21, 22, 23, 25, 36). In these previous studies, the micropores were all of the inactive type. This was also the case for those found within the oocysts of *E. brunetti* in our study. Within the oocysts of *E. brunetti* the largest number of micropores were observed in the stage with the largest surface area (the zygote).

Thus the micropore in *E. brunetti* is present in all stages of the life cycle except the microgamete. In the majority of cases, though, the micropore is present as a vestige rather than as an active micropore which is found only on actively growing organisms of the endogenous phase.

We are indebted to the *Central Veterinary Laboratory*, Ministry of Agriculture, Fisheries and Food, New Haw, Weybridge, Surrey, England, for supplying a pure sample of oocysts of *E. brunetti* and to *K. L. Fennestad*, V.M.D., Statens Seruminstitut, for provision and maintenance of the chickens. We gratefully acknowledge Mrs. *H. Ras* and Mrs. *J. Berg* for technical assistance, and Mrs. *A. Overgaard* and Mr. *F. Larsen* for photographic assistance.

The work was supported by grants from the *World Health Organization*, Geneva, the *Wellcome Trust*, the *British Council*, the *Danish Medical Research Council* and the *H. H. Ross Foundation*.

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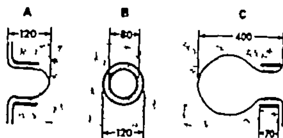
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DISCUSSION

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The exact number of micropores in the various developmental stages cannot be ascertained unless complete serial sections through individual organisms can be examined; this was not carried out. We have examined the relative number of micropores, *i.e.* the frequency with which one or more micropores were observed within the sections that were available through the different stages in the life cycle of the organism. It was found that the micropores were less frequent



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guinea pigs. If so, it was of further interest to examine if any difference in this activity could be found between the various LPS preparations, and to what extent this activity was comparable to that of a *Salmonella* LPS.

MATERIALS AND METHODS

Organisms

The bacterial strains used for isolation of LPS (endotoxin) were *Bacteroides fragilis* subspecies *fragilis* strain NCTC 9343 and E 323 *Bacteroides melaninogenicus* subspecies *intermedius* strain B 10 *Fusobacterium nucleatum* strain F1 and Fev 1 *Veillonella alcalescens* strain Ve 5 and *Leptotrichia buccalis* strain ... 9 In ... previous paper details about their isolation and cultivation have been described (11)

LPS ... isolated from whole or disintegrated (11) cells by the phenol-water extraction procedure (12) and further purified by ultracentrifugation. As reference endotoxin LPS from *Salmonella enteritidis* strain S-793 was used (generously provided by A C *Milner* Rocky Mountain Laboratory Hamilton, Mont., USA) The chemical and electronmicroscopic characterization of the LPS preparations have been reported (11)

Rabbit Polymorphonuclear Leukocyte

Polymorphonuclear leukocytes (PMN) were obtained from the peritoneal cavity of adult New Zealand White rabbits 8–10 h after intraperitoneal (i.p.) injection of 200 ml of ... solution containing 0.1 per cent glycogen (E. Merck AG Darmstadt, W-Germany) in sterile isotonic saline (0.85 per cent NaCl) to which 50 µg garamycin® (Schering Corporation, Bloomfield, N J USA) and 2.5 µg fungizone® (Squibb Flow Laboratories, Irvine, Scotland) per ml solution had been added Before harvesting the cells, the rabbits were bled thoroughly The ... p cavity was opened under aseptic conditions, and the exudate collected by rinsing the cavity with sterile isotonic saline containing 10 U of heparin (A/S Apothekernes Laboratorium for Specialpreparater Oslo) per ml The leukocyte-rich exudate was kept in an ice-bath until the isolation of the cells. After centrifugation of the exudate at 225 x g for 5 min (IEC, Universal Model UV) the cells were suspended in heparinized (10 U/ml) chilled Gey balanced salt solution (Gey' medium) containing 50 µg garamycin® and 2.5 µg fungizone® per ml. Erythrocytes present in the sediment were hemolyzed by adding ... solution of 0.83 per cent ammoniumchloride. After centrifugation, the PMN were resuspended in Gey' medium containing antibiotics and adjusted to ... 2 per cent solution with bovine serum albumin (BSA) (Armour Pharmaceutical Company Ltd., Eastbourne England) giving a final number of approximately 10 leukocytes per ml Ninety-two to ninety-four per cent of the leukocytes resisted staining with trypan blue All media had a pH 7.... 7.4 and were sterilized before use by passage through a filter with a pore ... of 0.45 µ (Millipore Filter Corp Bedford, Mass USA)

Measurement of Chemotactic Activity

As some of the LPS preparations were difficult to suspend in Gey medium, the stock solutions containing 2 mg ml were ultrasonicated (MSE/Mullard, 60 W ... 0 kc/) ... 0 C for 2 min. If necessary the pH was adjusted to 7.2 ± 0.2 with triethylamine The two compartments of a modified Boyden chamber ... ed (10) (Neuroprobe Bethesda, Md USA) were separated by Millipore filter (Millipore Filter Corp.) of pore size 3 µ. The lower compartment, having a volume of 0.... ml, was filled with the chemotactic medium, i.e. medium containing LPS and activated complement. Before placing the filter in the chamber 0.4 ml of the cell suspension was spun down (58 x g for 1.5 min) on the filter in a cytocentrifuge (Shandon Elliot, Cytospin SCA-0030) giving approximately 4 x 10⁶ PMN on the upper surface. The upper compartment, thus containing the leukocytes, was filled with Gey medium containing antibiotics and BSA. The chambers were then placed in ... moist incubator for 3 h at 37 C. Thereafter the filters were removed, stained and cleared according to *Boyden* (1962) before mounting them bottom side up with diatex (Diatex AB, Wilh. Becker Stockholm) on glass slides. PMNs which had completely crossed the intercompartmental filter during the incubation period were counted, using a 40 x objective and an 8 x ocular containing a micrognd (Leitz Wetzlar 10 x 10 mm) The average number of cells in 5 randomly selected high-power fields were counted, and the chemotactic activity expressed as the mean number of PMNs calculated from these fields.

Fresh pooled guinea pig serum stored at −25 C in 5 ml aliquots was used as the complement source. The serum was thawed at 4 C immediately before each experiment was performed. Aliquots of 1.5 ml from each stock solution of LPS containing 200 µg/0.1 ml were diluted two-fold. Dilutions thus containing 6.25 µg and 3.12 µg per 0.1 ml were tested for chemotactic activity Five mg/per cent casein (E. Merck AG) was used as the control for positive chemotaxis. To examine if contaminating glucans of some of the LPS preparations used might be responsible for the chemotactic activity exerted by these preparations, the chemotactic activity of glycogen was tested. Whether true chemotaxis was taking place or not was controlled by placing the same amount of LPS sus-

Acta path. microbiol. scand. Sect. B, 85 374–380 1977

RABBIT POLYMORPHONUCLEAR LEUKOCYTE MIGRATION *IN VITRO* IN RESPONSE TO LIPOPOLYSACCHARIDES FROM *BACTEROIDES, FUSOBACTERIUM* AND *VEILLONELLA*

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Sveen, K. Rabbit polymorphonuclear leukocyte migration *in vitro* in response to lipopolysaccharides from *Bacteroides Fusobacterium* and *Veillonella*. Acta path. microbiol. scand. Sect. B 85 374–380 1977

Purified lipopolysaccharides (LPS) from strains of *Bacteroides Fusobacterium* and *Veillonella* incubated with guinea pig serum, were tested for chemotactic activity against rabbit polymorphonuclear leukocytes (PMNs) in modified Boyden chambers. Comparisons were made to a *Salmonella* LPS (*S. enteritidis* S-795) Submicrogram amounts of LPS induced positive chemotaxis, and a typical dose-response relationship up to certain dose levels was observed. The difference in chemotactic activity between the *Veillonella* LPS and LPS-S-795 was not statistically significant. The *Fusobacterium* LPS showed either a non-significant or a highly significantly lower chemotactic capacity than LPS-S-795 The *Bacteroides* LPS were also clearly chemotactic, but considerable less when compared to the *Salmonella* LPS When serum was not added the LPS preparations showed no chemotactic activity

Key words *Bacteroides Fusobacterium Veillonella* lipopolysaccharides leukocyte chemotaxis Boyden chambers.

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Received 20.xii.76 Accepted 5 vii 77

Activation of the complement system by bacterial lipopolysaccharides (LPS) when incubated with serum from different species leads to the generation of a factor chemotactic for polymorphonuclear leukocytes (PMNs) (9) During this activation process there is a consumption of the terminal components of C (C3 through C9) and a relative sparing of the earlier acting components by LPS (3 4 8)

In examination of the activity of cytotaxins and cytotaxigens on PMNs, the Boyden chamber device has been extensively used. The aim of this study was, by use of a modified Boyden chamber device to examine if LPS purified from the anaerobic Gram-negative organisms of *Bacteroides fragilis Bacteroides melaninogenicus Fusobacterium nucleatum* and *Veillonella* were able to elaborate a factor chemotactic for PMNs when incubated in serum from non immunized

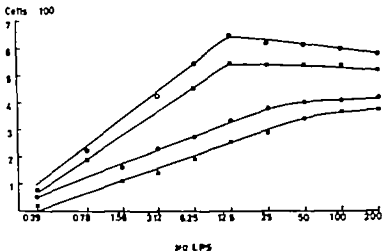


Fig. 1 Leukocyte chemotaxis of lipopolysaccharides isolated from the strains S-795, Ve 5, E 323 and Fev 1 incubated in guinea pig serum and tested in a modified Boyden chamber device. The abscissa represents the number of cells per high-power field on the chemotactic side of the filter disc. Each dose-response curve is drawn through the mean values of the number of cells per high-power field from two filter discs. –O– S-795, □ – V 5, –● E 323, – Fev 1

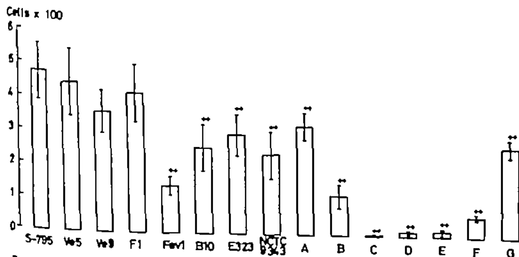


Fig. 2 Leukocyte chemotaxis of lipopolysaccharides isolated from strains of *Veillonella*, *Fusobacterium* and *Bacteroides* in the dose of 3.12 µg preincubated in guinea pig serum. The tests were performed with leukocytes from the same pool. For explanation of bacterial LPS and doses used see Materials and Methods. Ve 5 + serum A) not preincubated, B) preincubated at 56 C for 30 min, C) Ve 5 + Gey solution, D) undiluted serum, E) LPS-F1 + serum in both compartments, F) glycogen + serum, G) casein. Each column represent the mean number of cells per high-power field ± standard deviation (vertical bar) from six filter discs. LPS-S-795 was used for comparison. For statistical analysis the Wilcoxon rank test for two samples (two-tailed) was used. Values of $p \leq 0.05$ = statistically significant. $p > 0.05$ $^{**}p < 0.005$.

pension (3.12 μg/0.1 ml) in equal volume of undiluted serum in both compartments of the chamber. Alliquots of 1.5 ml of the LPS mixtures, containing equal volumes of LPS suspension and undiluted serum were preincubated in water bath at 37 C for 30 min and thereafter at 56 C for 30 min. The same was done with the controls, with the exception of casein. Controls were also included where the LPS-serum mixture was not preincubated or was preincubated at 56 C for 30 min. only

Statistical Methods

Standard deviation was calculated as previously reported (11). The statistical evaluation of differences of chemotactic activities was performed according to the Wilcoxon rank test for two samples (two-tailed) (2). The dose X on the dose-response curve was transformed to XT according to the formula $XT = \log (1 + X)$ and the Pearson correlation coefficient (7) was calculated according to the relationship between the transformed dose XT and the response Y.

EXPERIMENTS AND RESULTS

Incubation of LPS with guinea pig serum rendered the LPS-serum mixture chemotactically active on rabbit PMNs. When tested in ten fold and two-fold dilutions, submicrogram amounts of all LPS preparations induced a positive chemotactic activity for rabbit PMNs. As illustrated in Table 1 there was a considerable variation in the number of cells per high power field from one experiment to another. In these experiments, serum from the same pool was used while the PMNs were from different batches. When the dose response relationship was examined using two-fold dilutions of the different LPS preparations and plotted on a semi logarithmic paper each LPS exhibited its own specific curve. Up to a certain dose level the most active endotoxins showed a rather steep ascending dose-response relationship (Fig. 1). A maximum was usually found beyond which no further increase occurred but rather a diminution of the chemotactic response appeared. The less active endotoxins, *viz.* LPS-E 323 and LPS-Fev 1 gave increasing response in leukocyte migration also at high concentrations. The positive correlation coefficients were 0.99 for LPS-S-795 in the dose range 0.39 to 12.5 μg 0.98 for LPS-Ve 5 in the dose range 0.39 to 12.5 μg and 0.94 and 0.98 for LPS-E 323 and LPS-Fev 1 respectively in the dose range 0.39 to 200 μg. However the less active LPS preparations were also highly chemotactic to the PMNs in doses lying within the ascending part of the dose response curve from the most active ones. To avoid comparing the leukochemotactic activity of amounts of LPS lying on the decreasing part of the most active endotoxins and in order to spend minimal amounts of the LPS preparations, doses of 3.12 and 6.25 μg were chosen for a comparison with the chemotactic effect of LPS-S-795.

TABLE 1 *Leukocyte Chemotaxis in vitro of LPS Isolated from* V. alcalescens *Strain Ve 5*

Amount of LPS Ve 5 (μg)*	Cells per high-power field		
	Exp. 1 n = 2	Exp. 2 n = 2	Exp. 3 n = 2
100	573–685	69?–768	612–680
10	399–523	644–712	528–6?2
1	306–406	519–613	279–307
0.1	80–88	421–459	134–190
0.01	65–75	332–358	91–121
0.001	35–63	104–140	38–58
Serum alone	13–39	17–31	9–19
Gey's sol. + 10 μg LPS	0–2	3–5	?–4
Gey's sol. + serum**	15–27	14–20	3–13

* Reaction mixture: Equal volumes of LPS sonicated in Gey's medium and guinea pig serum.
** Equal volume of each.

Using 3.12 μg of LPS the *Veillonella* LPS and LPS-F 1 displayed no statistically significant difference in chemotactic activity from LPS-S-795 (Fig. 2). The difference between the LPS-S-795 and the other endotoxins or the controls was, however highly significant ($p < 0.005$). LPS in Gey's solution gave significantly lower cell counts per high power field than serum alone. No difference was observed in the response when the amount of LPS was increased nor when no LPS was added to Gey's solution. Glycogen incubated with guinea pig serum induced only slight

ned may account for this finding. These re sults indicate that comparisons of chemotac tic activity from different doses of test substances should be made with cells from the same batch.

The decrease in number of cells on the chemotactic side of the filter when high doses of LPS were employed, may be due to the migration of PMNs into the chemotactic me dium. Preliminary experiments showed that approximately ten per cent of the cells fell off the lower surface of the filter during the incubation period

The results from the preincubation of LPS in Gey's solution demonstrate that bacterial lipopolysaccharides have no direct leukochemotactic activity The short time during which heat-stable leukochemotactic media tors are elaborated at the optimal tempera ture before the heat-labile serum components are inactivated, may account for the low cell counts found when the LPS-serum mixture was preincubated at 56 C for 30 min before testing on chemotactic activity The significantly lower cell count found when no preincubation of the endotoxin-serum mixture was performed in contrast to when preincubated at 37 C for 30 min indicates that there definitely is a time lag before a gradient of chemotactic mediators is built up Furthermore, incubation at 37 C only renders an unspecific and uncontrolable activation of heat-labile inhibitors or activators of the serum which may influence the chemotactic activity on the PMN The interaction of LPS with guinea pig serum preincubated at 37 C for 30 min and thereafter at 56 C for 30 min thus demonstrated the formation of heat stable cytotaxins. The cytotaxinogenic activity was different for each LPS tested. The chemotactic activity induced by *Veillonella* LPS was comparable to that of the *Salmonella* and may thus reflect a similarity between their lipid moiety lipid A (6) The lipid A of the *Fusobacterium* LPS has also been found to be similar to that of the *Salmonella* LPS (5) It was, therefore, unexpected to find such a low chemotactic activity of LPS-Fev 1 since the activity of the LPS-F1 was comparable to that of the *Salmonella* LPS. The dispersion of the LPS complex of Fev 1 however may have been poor and thus have accounted for the low chemotactic activity found. *Bacteroides* LPS seem to be quite different from the *Salmonella* LPS as they do not seem to have a typical lipid A (*T Hofstad* personal communication) Such a difference in macromolecular composition may thus explain both the low chemotactic capacity and the low endotoxic activity found in other biological tests (11)

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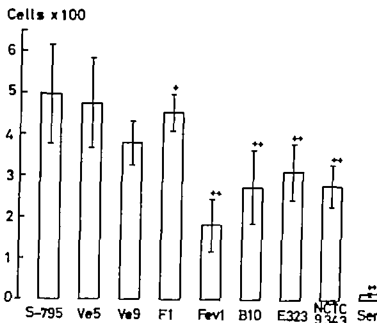


Fig 3 Leukocyte chemotaxis of lipopolysaccharides from the same bacterial strains as shown in Fig. 2, *incubated in guinea pig serum in the dose of 6 25 µg.* For comparison statistical analysis and probability see legend to Fig 2 Each column represents the mean number of cells per high-power field ± standard deviation (vertical bar) from six filter discs. Ser = serum. $^{+}p > 0.05$ $^{++}p < 0.005$

chemotactic activity One microgram of glycogen in the reaction mixture, corresponding approximately to the content of neutral sugars in 3 12 µg of LPS from B10 and NCTC 9343 gave PMN counts averaging 60 per high power field. Glycogen alone had no chemotactic effect on PMNs

When the LPS-serum mixture was neither preincubated at 37 C nor subsequently at 56 C, approx. 70 per cent of the chemotactic activity was found. When the same test suspension was preincubated at 56 C before placing it in the test chambers, only about 25 per cent leukochemotactic effect was elaborated. No demonstrable or only weak chemotactic activity was exerted on PMNs when both compartments of the modified Boyden chamber contained equal volumes of the reaction mixture.

When 6.25 µg of each LPS preparation were tested (Fig 3) the chemotactic effect was insignificantly higher than when 3 12 µg of the same LPS was examined. However the activity of the various LPS was ranked in the same order in both types of experiment.

DISCUSSION

The modified Boyden chamber technique provided a sensitive recording of chemotaxis since only submicrogram amounts of LPS were necessary for the attraction of PMNs. This is in agreement with findings reported by *Snyderman et al* (1968) testing *Veillonella* endotoxin and using the same method.

The most variable component of this experimental system is by far the PMNs, to which the variable results referred to in Table I may be ascribed The cells of a batch consist of individual cells with different age and functional capacity and their chemotactic response may vary accordingly An insignificant difference in chemotactic activity was found when two successive two-fold dilutions from the ascending part of the dose-response curve of the LPS preparations were studied separately (cf Fig 2 and 3) This disagrees considerably with the results obtained when two-fold dilutions were tested in the same experiment (cf Fig 1) Since serum from the same pool and LPS from the same two-fold dilution were employed in all experiments the different batches of cells

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RABBIT POLYMORPHONUCLEAR LEUKOCYTE MIGRATION *IN VIVO* IN RESPONSE TO LIPOPOLYSACCHARIDES FROM *BACTEROIDES*, *FUSOBACTERIUM* AND *VEILLONELLA*

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Sveen, K. Rabbit polymorphonuclear leukocyte migration *in vivo* in response to lipopolysaccharides from *Bacteroides*, *Fusobacterium* and *Veillonella*. Acta path. microbiol. scand. Sect. B, 85: 381-387, 1977.

Subcutaneously implanted chambers in rabbits were used for testing the migration of polymorphonuclear leukocytes in response to injected LPS isolated from strains of *Bacteroides*, *Fusobacterium* and *Veillonella*. A *Salmonella* LPS was used as reference endotoxin. No difference in chemotactic activity between the *Veillonella* LPS and LPS from *Salmonella* was found. *Fusobacterium* LPS showed insignificantly lower chemotactic capacity than the *Salmonella* LPS. The *Bacteroides* LPS were all significantly less chemotactic than the reference endotoxin. An insignificant correlation between the amount of exudate aspirated from the chambers 5 h after injection of the different LPS preparations and the number of leukocytes per µl of exudate was found.

Key words: *Bacteroides*; *Fusobacterium*; *Veillonella*; lipopolysaccharides; leukocyte chemotaxis; wound chambers.

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Received 20.xii.76. Accepted 9 77

Initiation of inflammation through accumulation of polymorphonuclear leukocytes (PMNs) at the injection site is a characteristic feature following administration into animals of lipopolysaccharides (LPS) isolated from Gram negative bacteria. This has, therefore, made LPS (endotoxin) a valuable tool in studies of the inflammatory response.

The production in rabbits of primary skin inflammation and the local and generalized Shwartzman reaction of LPS isolated from *Veillonella alcalescens*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Bacteroides fragilis* and *Bacteroides melaninogenicus* has been reported (9). It has also been demonstrated that LPS from these microorganisms, when incubated in guinea pig or rabbit serum, elaborate factors chemotactic for rabbit PMNs in vitro (8).

The present paper deals with the migration of PMNs *in vivo* using wound chambers (10). LPS from the same bacterial organisms as mentioned above was used. It was of

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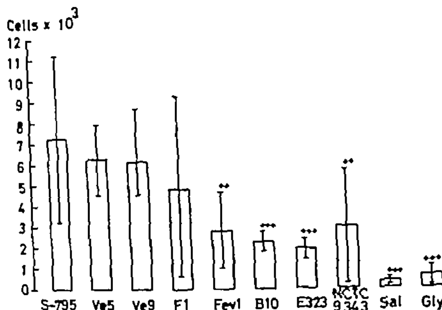


Fig. 1 PMN accumulation in wound chambers induced by lipopolysaccharides isolated from strains of *Veillonella*, *Fusobacterium* and *Bacteroides*. For explanation of coded lipopolysaccharides used see Materials and Methods. Doses of 12.5 µg of each LPS preparation and of glycogen was tested. Each column represents the mean number of cells per µl of wound fluid ± standard deviation (vertical bar) from 7 chambers 5 h after injection of test suspensions. Sal = saline, Gly = glycogen. Comparisons are made to LPS-S-795. For statistical analysis the Wilcoxon two-sample (two-tailed) test was used. Values of $p \leq 0.05$ were accepted as statistically significant. + $p > 0.05$, ++ $p < 0.02$, +++ $p < 0.005$

between the animals could be found a comparison of the total number of cells in the contents of fluid in all wound chambers in the different rabbits was included.

The volume of exudate aspirated from the different chambers at time zero, i.e. six days after the implantation, ranged from 337 ± 127.1 to 494.2 ± 132.1 µl. The volume of exudate aspirated at time zero from the chambers to be inoculated with LPS-S-795 (337.8 ± 127.1 µl) was compared to the volume of exudate in each of the other test chambers (356.6 ± 120.4 to 494.2 ± 132.1 µl). The difference was not statistically significant.

The volume of fluid aspirated from the chambers 5 h after injection of the test suspensions, which ranged from 260.7 ± 68.1 to 390.0 ± 106.3 µl, was not different from that from the chambers injected with the reference endotoxin which measured 293.7 ± 19.1 µl ($p > 0.05$). Thus considerable less wound chamber fluid was aspirated after 5 h, than at time zero. No significant correlation between the amount of exudate induced by the various LPS preparations and the number of leukocytes per µl of the exudate was found ($r = 0.62$, $p > 0.1$).

At time zero there was no difference regarding the number of leukocytes per µl of exudate in the chambers to be inoculated with different test substances (ranging from 408.5 ± 92.2 to 457.1 ± 76.1 cells) when compared with those to be injected with LPS-S-795 (392.8 ± 109.6 cells) ($p > 0.05$). At the end of the experiment (Fig. 1) no difference in the number of leukocytes per µl of exudate was found when the *Veillonella* LPS and LPS-F 1 induced wound chamber fluid was compared to that induced by the reference endotoxin ($p > 0.05$). The other endotoxins induced less *influx of* PMNs than

particular interest to examine if the *Bacteroides LPS* which in *in vitro* investigations showed a very low activity induced PMN migration *in vivo* and if so to what extent when compared with LPS from other anaerobic microorganisms and from *Salmonella*

MATERIALS AND METHODS

Microorganisms

The strains used for isolation of LPS were *Bacteroides fragilis* subspecies *fragilis* strains NCTC 9343 and E 323 *Bacteroides melaninogenicus* subspecies *intermedius* strain B10 *Fusobacterium nucleatum* strains F1 and Fev 1 *Veillonella alcalescens* strain Ve5 and *Veillonella parvula* strain Ve9 Details concerning their isolation and cultivation have been reported (9) LPS was isolated from whole or disintegrated cells (9) by extraction with phenol-water (12) and purified by ultracentrifugation and treatment with ribonuclease and deoxyribonuclease (9) LPS from *Salmonella enteritidis* S-795 (reference endotoxin) was kindly provided by K C Milner (Rocky Mountain Laboratory Hamilton, Montana, USA) The chemical composition of the LPS preparations and their electron microscopical appearance have been reported (9)

Measurement of Leukocyte Migration

Five sterile wound chambers were implanted subcutaneously on each lateral side of six-months-old New Zealand White rabbits as described earlier (10) Six days after implantation, the exudate formed was aspirated and immediately chilled to 0 C. LPS and control solutions were then injected into the chambers as described in Experiments and Results. The amounts of exudate collected from each chamber before and 5 h after injection of LPS and control solutions were measured and 25 µl of it immediately transferred to 475 µl of methylene blue to determine the number of leukocytes by counting in a Burker chamber For differential counts, smears of the exudate prepared on glass slides were stained with May-Grünwald Giemsa (Pappenheim staining)

Stock solutions of LPS containing one mg per ml, were prepared in sterile isotonic saline (0 85 per cent) All stock solutions were treated ultrasonically (MSE/Mullard, 60 W 20 kc/s) at 0 C for 2 min. If necessary the pH was adjusted to 7 2 ± 0 2 using triethylamine

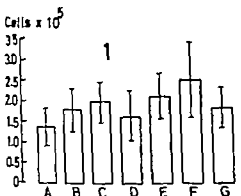
Statistical Methods

Standard deviation was calculated as previously reported (10) The Wilcoxon rank test for two samples (two-tailed) (3) was used for the evaluation of the differences in chemotactic activities exerted by LPS-S-795 and the other endotoxins. The same test was also used for calculating the statistical significance of differences in the responses of rabbits to the test solutions. For the evaluation of the correlation between amount of exudate and number of cells per µl of exudate, the Spearman coefficient of rank correlation was used (3)

EXPERIMENTS AND RESULTS

In preliminary experiments two-fold dilutions of the LPS preparations were tested. When LPS was applied up to a given amount to the granulation tissue within the chambers, a typical dose response relationship was seen. This was characteristic for each of the LPS preparations and varied from 25–200 µg The dose levels in the ascending part of the dose response curves varied according to the high or low chemotactic activity exerted by the various LPS as also has been reported previously (10) When the dose-response curves of the different preparations were compared, 12.5 µg of the LPS was found to lie within the ascending part of all dose response curves and was, as previously discussed (8) chosen for the comparison of the chemotactic activity of the LPS preparations from anaerobic organisms with that exerted by LPS-S-795

LPS in doses of 12.5 µg in 0.4 ml of isotonic saline from each of the eight different LPS preparations were injected into eight chambers on each rabbit In the remaining two chambers 12.5 µg of glycogen (E. Merck AG Darmstadt W-Germany) in 0 4 ml of saline and 0 4 ml of saline alone were injected as controls, respectively Glycogen was included since LPS-NCTC 9343 and LPS-B10 contain large amounts of neutral sugars (9) The sites of the implanted chambers were numbered, and the endotoxin preparations or controls were not injected into the same chamber more than once The comparative examinations consisted of measurements of the volume of exudate in the chambers before and 5 h after injection of LPS the number of leukocytes per µl of exudate and the total number of leukocytes within the chamber To examine if any biological variance



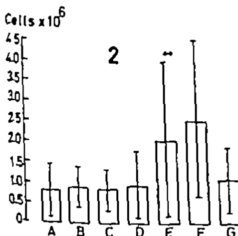


Fig 2 Total number of leukocytes in the chambers of each rabbit 6 days after implantation (1) and 5 h after injection of different bacterial lipopolysaccharides and controls (2). For explanation of statistical analysis and probability see legend to Fig 1. Rabbit A was chosen at random for comparison. Each column represents the mean number of cells in the wound fluid from ten chambers ± standard deviation (vertical bar). $^{+}p > 0.05$ $^{++}p < 0.05$ $^{+++}p < 0.01$

calculated in all the wound chambers in the individual rabbits at time zero and at the end of the experimental period. Higher numbers of cells were found in the wound fluid in the chambers of rabbits E and F both before and after the injection of the different LPS and controls. The higher number of cells in the wound chamber fluid of these two animals at the end of the experimental period was found to be induced by all LPS preparations as well as by control substances.

The relative distribution of PMNs and mononuclear leukocytes (MNs) in the exudate at time zero and 5 h after application of test preparations was in accordance with previous result (10). In the LPS induced wound chamber fluid an average of 94 per cent of the cells were PMNs as compared to about 75 per cent in that induced by saline. The higher number of MNs in the saline induced wound chamber fluid than that found in the LPS induced fluid was highly significant ($p < 0.005$).

DISCUSSION

When LPS isolated from strains of *Bacteroides*, *Fusobacterium* and *Veillonella* is applied on a newly formed granulation tissue within chambers subcutaneously implanted in rabbits, this results in an accumulation of polymorphonuclear leukocytes.

The *in vivo* chamber method measures a more complex phenomenon than the *in vitro* Boyden chamber method does. Thus factors like capillary flow, capillary permeability, mobilization of PMNs from blood depots as well as the rate of PMN production ought to be considered. However, pilot studies concerning fractionations of the wound chamber fluid on Sephadex columns suggest that LPS *in vivo* as well as *in vitro* promote chemotaxis through activation of the complement system.

Preliminary experiments showed that the elaboration of chemotactic mediators also seem to depend on the physical state of the LPS since sonical treatment enhanced the attraction of PMNs. This is in accordance with the increased capacity of LPS, when sonicated, to produce skin inflammatory reactions in rabbits (10) and to attract PMNs *in vitro* (8).

The results from this study agree well with the *in vitro* studies using the modified Boyden chamber device (8). Thus the relative migratory activity induced by the various LPS showed the same pattern in the *in vitro* as

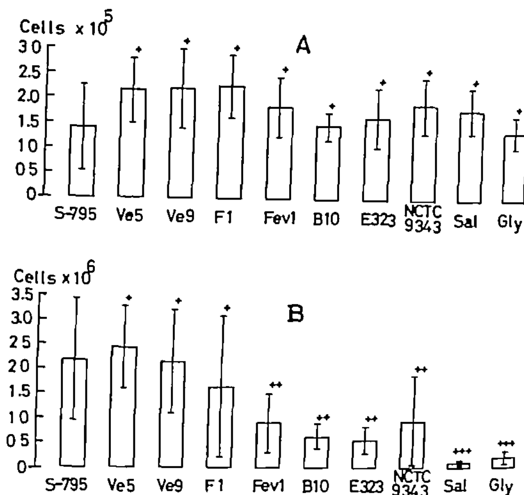


Fig 2 Total number of leukocytes in th chambers 6 days after implantation (A) and 5 h after injection of bacterial lipopolysaccharides (B) For explanation of coded test suspensions used, comparison, statistical analysis and probability see legend to Fig 1 + $p > 0.05$ ++ $p < 0.05$ +++ $p < 0.005$

did LPS-S-795 The difference in number of leukocytes in the LPS-Fev 1 and NCTC 9343 induced chamber fluid was at the two per cent level Wound fluid from chambers injected with LPS-B10 and LPS-E 323 as well as with glycogen showed significant leukocyte counts that were only about one third one fourth and one eleventh of that induced by LPS-S-795 ($p < 0.005$) The activity of LPS-NCTC 9343 was significantly higher than glycogen ($p < 0.005$) LPS-B10 was also more active than glycogen, but less pronounced than LPS-NCTC 9343 was ($p < 0.01$)

As shown in Fig 2 there was no statistically significant difference in the total number of leukocytes accumulated in the different test chambers at time zero the mean ranged from 0.140 ± 0.087 to $0.225 \pm 0.063 \times 10^6$ cells per chamber Five hours after the application however the difference between the total content of leukocytes in the LPS-S-795 induced wound chamber fluids and that induced by *Bacteroides* LPS as well as LPS-Fev 1 was statistically significant ($p < 0.05$) as was the difference between the LPS-S-795 and saline or glycogen ($p < 0.005$) The number of cells accumulated in the exudate induced by *Veillonella* LPS and LPS-F1 was similar to that of the reference endotoxin ($p > 0.05$)

The total number of PMNs induced by LPS-B10 and LPS-NCTC 9343 was three and four times higher respectively than that induced by glycogen ($p < 0.005$)

Fig. 3 shows the total number of leukocytes

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well as in the *in vivo* experiments. This finding indicates that PMN migration *in vivo* also is due to chemotaxis. Thus the chemotactic effect of the *Veillonella* LPS either expressed as leukocytes per µl of exudate (cf Fig 1) or as number of cells per wound chamber (cf Fig 2) was comparable to that of the *Salmonella* LPS whereas the *Fusobacterium* LPS either was similar or lower in activity. The present study also shows that the *Bacteroides* LPS posses low chemotactic effect on leukocytes when compared to the other LPS and to the *Salmonella* LPS. As discussed in a previous paper (8) difference in macromolecular composition may account for the low chemotactic activity of *Bacteroides* LPS. The *Veillonella* LPS and the *Fusobacterium* LPS have a molecular composition comparable to that of the *Salmonella* and this may thus explain their high chemotactic activity.

The direct or indirect effect of LPS when applied on newly formed granulation tissue is an efflux of cells as well as an exudation of fluid probably due to an increased permeability of the vessels of this tissue. The correlation between the amount of wound chamber fluid induced by the different LPS preparations and the number of leukocytes per µl of the fluid, however was insignificant ($p > 0.1$). This indicates that an LPS with a high chemotactic activity on PMNs not necessarily is tantamount to a large amount of exudate formed.

A significant variance between the rabbits in the response to the different LPS and controls was found (cf Fig 3). In the model used however this does not influence the comparison of chemotactic activity exerted by the different LPS since they all were tested simultaneously in the same rabbits.

The *in vivo* chamber method has several advantages over the modified Boyden chamber method: the migration of PMNs as well as MNs may be studied and compared, information about the vascular permeability and the formation of exudate in connection with the inflammatory response may be gained during a defined period of time, and the exudate may be subjected to chemical and biological examinations.

Chemotaxis physiologically occurs as part of an inflammatory response. A possible connection between chemotaxis and gingivitis has been postulated by *Tempel et al.* (1970). PMNs are thus constantly present both in the gingival crevices and within the junctional epithelium of healthy as well as chronically inflamed gingiva (1 2 6).

A gradual increase in the number of PMNs within the gingival crevices during the progress of gingivitis has been observed (2). It has also been shown that bacteria *per se* are chemotactic (4) and that oral bacteria produce factors directly chemotactic for PMNs *in vitro* (11). Furthermore, human dental plaque material has been found to possess chemotactic activity for neutrophil leukocytes *in vivo* (5). Since endotoxin may be liberated from disintegrating Gram-negative bacteria of the human gingival plaque and the quantity of endotoxin in the gingival exudate can be determined (7) it seems likely that LPS by interaction with factors present in the plasma may contribute to the destruction of tissue in patients suffering from periodontitis.

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des were chemotactic for rabbit polymorphonuclear leukocytes in vitro (20) and in vivo (21). In these test systems the endotoxic activity of the *Bacteroides* LPS was very low compared to that of the other LPS and LPS of *Salmonella enteritidis* which was included for comparison. The comparative studies of the endotoxicity of LPS prepared from the above Gram-negative anaerobes have therefore been extended to include lethality in mice and 11-days-old chick embryos, pyrogenicity in rabbits and the ability to gel lysates of *Limulus polyphemus* amoebocytes.

Animal species vary in their susceptibility to the lethal effects of endotoxins. Mice are relatively resistant. Chick embryos, on the other hand, are extremely susceptible to endotoxin between 8 and 12 days of incubation (5). The most studied and also the most typical and distinguishing host reactive property of an endotoxin is the biphasic fever occurring after intravenous injection. With regard to sensitivity the *Limulus* assay of Levin & Bang (1968) is superior to all other tests for endotoxicity. In fact, this assay has been used to detect endotoxemia in patients suffering from *B. fragilis* sepsis (18).

MATERIALS AND METHODS

Bacterial Strains and Isolation of LPS

The strains used for preparation of LPS were LiF E 323 *Bacteroides melaninogenicus* ss. intermedius E 323, *Bacteroides melaninogenicus* ss. intermedius B 10, *Fusobacterium nucleatum* F1 and Fn 1, *Veillonella alcalescens* V 5 and *Veillonella parvula* V 9. The origin of the strains, method for cultivation, and extraction of whole or crushed cells with 45 per cent aqueous phenol has been reported (19). LPS from *Salmonella enteritidis* strain 8-795 was used as reference endotoxin.

Bioassays

Mouse lethality. Mice of both sexes of the strain NMRI weighing 20 ± 1 gram were used. The animals were fed commercial pellets and water ad lib and kept at a temperature of 20–22° C and relative humidity of 45–50 %. In order to obtain good dispersable suspensions, stock solutions of LPS in sterile isotonic saline were sonicated at 0° C before use (MSE/Mullard, 60 W 20 kc/s). Two-fold dilutions of LPS in saline were injected intraperitoneally (i.p.) into groups of six mice, each mouse receiving 0.9 ml of the dilution. Two-fold dilutions of the LPS preparations containing 12.5 µg of actinomycin-D® (AM D) (Merck, Sharp and Dohme Div. of Merck & Co. Inc., West Point, Pa. 19486 USA, lot 449 N) were likewise injected into groups of six mice. Mice injected with sterile saline and saline containing 12.5 µg of AM D served as controls. Deaths were recorded daily for a period of three days after inoculation.

Lethality for chick embryos. The capacity of the LPS to kill chick embryos was examined according to *Milner & Finkelstein* (1966). Eleven days after incubation of White Leghorn eggs in a self-turning, humidified incubator at 37° C, the chick embryos were inoculated intravenously (i.v.) with different doses of LPS agitated well in pyrogenfree saline (5) and returned to the incubator. Each dilution of the LPS preparation was tested in groups of ten embryos. Saline alone served as negative control. Lethality was recorded 24 and 48 hours after inoculation.

Pyrogenicity in rabbits. The test was performed as described by *Milner & Finkelstein* (1966). New Zealand White rabbits of both sexes, weighing 2–3 kg, were used. They were caged separately and maintained in a quiet room at a constant temperature of 21° C. Three different doses of each LPS were tested employing eight rabbits at each dose level. Stock solutions of the different LPS were made in pyrogen-free saline and sonicated before each dilution was made. The saline diluent served as control. Samples of 2.0 ml were administered in the marginal ear vein. Copper-constantan thermocouples implanted rectally registered the temperature which was recorded automatically with a Honeywell Multipoint Recorder every 12 min for a period of six h after inoculation (23). When the fever curves did not return to the base line at the end of six h, they were extrapolated to that point.

Limulus gelation test. Limulus amoebocyte lysate (LAL) was prepared from adult female horseshoe crabs (*Limulus polyphemus*) as described by *Hochstein et al.* (1973) using distilled water for lysis of the amoebocytes. Pyrogen-free glassware was prepared by soaking it in strong chromsulfuric acid bath for 24 h, rinsing it in re-distilled and thereafter in pyrogen-free water. Then it was wrapped in pyrogen-free aluminum foil and baked in a dry oven at 150° C for five h. The LPS preparations were sonicated in pyrogen-free re-distilled water. The stock solutions all had a pH in the region 6.0–8.0, which is necessary for performing the LAL test. Serial two-fold dilutions were made and between each dilution the diluted endotoxin was agitated for two minutes.

The LAL test was performed by incubating 0.1 ml of test solution with 0.1 ml of LAL in Pyrex glass tubes, 10 mm in diameter × 75 mm long

Acta path. microbiol. scand. Sect. B 85 388–396 1977

LETHALITY FOR MICE AND CHICK EMBRYOS, PYROGENICITY IN RABBITS AND ABILITY TO GELATE LYSATE FROM AMOEBOCYTES OF *LIMULUS POLYPHEMUS* BY LIPOPOLYSACCHARIDES FROM *BACTEROIDES, FUSOBACTERIUM* AND *VEILLONELLA*

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National Institutes of Health National Institute of Allergy and Infectious Diseases,
Rocky Mountain Laboratory Hamilton Montana 59840 USA

Sveen, K., Hofstad T and Milner K. C. Lethality for mice and chick embryos, pyrogenicity in rabbits and ability to gelate lysate from amoebocytes of *Limulus polyphemus* by lipopolysaccharides from *Bacteroides Fusobacterium* and *Veillonella* Acta path. microbiol scand. Sect. B 85 388–396 1977

Phenol water extracted lipopolysaccharides (LPS) from *Veillonella Fusobacterium nucleatum Bacteroides fragilis* and *Bacteroides melaninogenicus* were lethal for mice and 11-days-old chick embryos, pyrogenic in rabbits, and gelated *Limulus* amoebocyte lysate. Mouse lethality was considerably enhanced by actinomycin D In all test systems the endotoxin activity of *Veillonella* and *Fusobacterium* LPS was comparable to that of LPS from *Salmonella enteritidis* which was included as a reference endotoxin. The endotoxicity of the *Bacteroides* LPS was very low While nanograms of the *Veillonella* and *Fusobacterium* LPS killed the chick embryos and gelated the *Limulus* lysates, microgram amounts of the *Bacteroides* LPS were needed to give positive reaction in the same test systems. As much as 74 μg of the most active *B fragilis* LPS were required to give a typical biphasic fever response in rabbits. A significant correlation was found between all test results ($r = 0.90$–0.98 $p < 0.001$)

Key words *Veillonella F nucleatum B fragilis B melaninogenicus* lipopolysaccharides lethality pyrogenicity Limulus test

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Received 23 i 77 Accepted 27 v 77

Phenol/water extracted lipopolysaccharides (LPS) from *Bacteroides fragilis Bacteroides melaninogenicus Fusobacterium nucleatum Veillonella alcalescens* and *Veillonella parvula* produced primary inflammatory skin reactions as well as local and generalized Shwartzman reactions in rabbits (19) Also,

AM-D would kill 15–20 per cent of the mice within a period of three days.

Mice inoculated i p with lethal doses of LPS alone, or in combination with AM D became ill within one h. They did not eat or drink water and displayed uncoordinated movements, and most had diarrhea or uncontrolled stool. The hind part became paralytic, and shortly before death the mouse was cyanotic with labored breathing

Lethality for Chick Embryos

Intravenous injections of LPS into eleven-days-old chick embryos proved these to be highly susceptible (Table 2) The fifty per cent lethal end-points ($CELD_{50}$) of LPS from the *Fusobacterium* strains and from the *V alcalescens* strain Ve 5 were close to that of LPS prepared from *S enteritidis* S-795 LPS from the *V parvula* strain Ve 9 and from the *B. fragilis* strain E 323 showed lower activity LPS from the *B. melaninogenicus* strain B 10 and *B fragilis* strain NCTC 9343 were the least active

Pyrogenicity in Rabbits

Normal, unimmunized rabbits reacted to all LPS preparations with a febrile response. Calculations of the FI_{40} showed considerable differences in activity among the different endotoxins (Table 3) LPS-Ve 5 was 12 times less active that the reference endotoxin

TABLE 2 *Lethality for 11-days-old Chick Embryos of LPS Isolated from* Veillonella, Fusobacterium *and* Bacteroides

Source of LPS	Chick embryo LD_{50} (µg)	Rank	Ratio
S 795	0 0037	1	1 0
Ve 5	0 0109	3	2.9
V 9	0.1077	5	29 1
F1	0 0088	2	2 4
Fev 1	0 0185	4	5 1
B 10	0 9100	8	245.9
E 323	0.1925	6	52.0
NCTC 9343	0 8900	7	240.5

Comparison made to LPS-S-795

TABLE 3 *Pyrogenicity in Rabbits of LPS Isolated from* Veillonella, Fusobacterium *and* Bacteroides

Source of LPS	FI_{40} (µg)	Rank	Ratio[b]
S-795	0 064	1	1.0
Ve 5	0 770	2	12.0
Ve 9	9.200	3	143 7
F1	7.800	4	121 9
Fev 1	6 800	5	106.2
B 10	150 000	7	2343 8
E 323	74 000	6	1156.3
NCTC 9343	700 000	8	10937.5

Fever index of 40 cm²
[b] Comparison made to LPS-S-795

LPS from F1 Fev 1 and Ve 9 were 9 12 times less active than LPS-Ve 5 again. LPS from all *Bacteroides* strains displayed an extremely low pyrexial activity as they showed an effect varying from approximately 1 1000 to 1 10,000 of that from the LPS-S-795 Ac

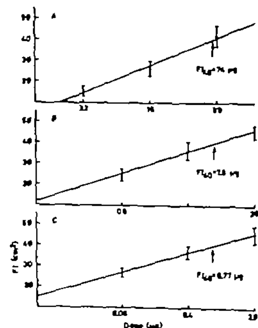


Fig 1 Graphic estimation of FI_{40} of LPS prepared from (A) *B fragilis* strain E 323 (B) *F nucleatum* strain F1 and (C) *V alcalescens* strain Ve 5 Six to eight rabbits were used per dose level Vertical bar indicates range of 1 standard error of each mean

capped with pyrogen free aluminium foil at 37 Pyrogen-free water and LAL, as well as LAL alone, served as negative controls. The former served as an evidence that water used for dilutions was endotoxin free, the latter that the equipment used was pyrogen free Positive tests were those that remained adherent to the bottom of the test tube as a firm opaque gel after one h when the test tube was inverted carefully at 180 C. Recorded were also the end points showing semisolid or soft gel formation, the clot being broken up by inverting the test tube Increased viscocity only was recorded as a negative reaction

Statistical Methods

Mouse mean lethal dose (MLD_{50}) and chick embryo mean lethal dose ($CELD_{50}$) were estimated by the method of *Kärber* (1931) The fever index (FI) was calculated as reported by *Rudbach et al* (1976) The gelation capacity of the different LPS preparations on *Limulus* lysate was calculated from samples in duplo The activity of the endotoxins in each test was ranked with the most active ranked as 1 Analysis was performed by using the Spearman rank correlation coefficient (3)

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The chemical composition of the LPS preparations have been reported (19)

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All LPS preparations were lethal for mice (Table 1) No marked differences were found between the lethality of LPS prepared from the *Veillonella* and the *Fusobacterium* strains and that of the reference endotoxin prepared from *S enteritidis* S-795 In contrast, the lethal effect on mice of LPS from the *Bacteroides* strains were very low The enhancing effect of 12.5 µg of AM D on the lethal activity of the different LPS preparations varied from 697 to 1189 times. The difference in lethal activity between the reference endotoxin and the different LPS preparations was more pronounced when AM D was added than without the addition. Thus, the less enhancing effect of AM D on the different LPS preparations when compared with LPS-S-795 ranged from 2 6 to 41.5 per cent. The addition of AM D to the LPS did not rank the LPS preparations in the same order of activity since LPS-E 323 was found more active than LPS-B 10 when AM D was not added Actinomycin D in the dose used produced no mortality whereas, according to preliminary experiments, a dose of 25 µg of

TABLE 1 *Lethality in Mice Following Intraperitoneal Administration of LPS Isolated from Veillonella, Fusobacterium and Bacteroides, without or with 12.5 µg Actinomycin D Added*

Source of LPS	Mouse LD_{50} (µg of LPS) without AM-D	AM D added	E factor	Ratio		Enhancement (%) of AM D[d]	Rank
S enteritidis S-795	270 0	0.227	1189	1 0	1 0	–	1
V alcalescens Ve 5	1,359 4	1.395	974	5.0[b]	6.1	18.1	3
V parvula Ve 9	2,250 0	3.228	697	8 3	14 1	41 4	5
F nucleatum F1	1 195.5	1.339	893	4 4	5.9	24 9	2
F nucleatum Fev 1	1 430 0	1 716	833	5.3	7 6	29.9	4
B melaninogenicus ss. *intermedius B 10*	21 937.5	18.949	1158	81.3	83 5	2.6	7
B fragilis ss. *fragilis* E 323	17 447 0	24.219	720	64 6	106 7	39 4	6
B fragilis ss. *fragilis* NCTC 9343	28 666 0	34,375	834	106 2	151 4	29.9	8

Enhancement factor of actinomycin-D
b Ratio to LPS S-795 based on LD_{50} without antibiotic added
Ratio to LPS-S-795 based on LD_{50} when antibiotic added.
d The percentage higher enhancing effect of AM D on LPS S-795
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Ve 5	0.0109	3	2.9
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Ve 5	0.770	2	12.0
Ve 9	9.200	5	143.7
F1	7.800	4	121.9
Fev 1	6.800	3	106.2
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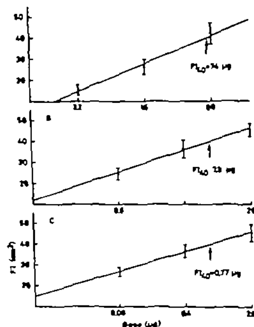


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Ratio to LPS-S-795 based on LD_{50} when antibiotic added
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The lethal effect of the LPS preparations without antibiotic added ranked

TABLE 5 *Comparison of the Sensitivity of Different Tests for Endotoxic Activity of LPS Isolated from* Veillonella, Fusobacterium *and* Bacteroides. *The Results of the LAL Test are Used for Calculating the Ratio*

Source of LPS	Pos th. LAL test (ng of LPS)	Ratio (LAL = 1)		
		$CELD_{50}$[b]	MLD_{50}	FI_{40}[d]
S-795	7.616	—2.1	35 4	8 4
Ve 5	9 138	1 1	148.7	84 2
Ve 9	24 371	4 4	92.3	377 4
F1	9.138	—0 9	130.8	853.5
Fev 1	36 562	—0.5	39 1	185 9
B 10	585.937	1.5	37 4	256.0
E 323	146 484	1.3	119 1	503 2
NCTC 9343	781.250	1 1	36.7	896.0

The LAL test was less sensitive

[b] Chick embryo LD_{50}.

Mouse LD_{50} without AM D added to LPS suspension.

[d] Fever index of 40 cm²

the bottom of the test tube was used, LPS-S-795 LPS-Fev 1 and LPS-F1 were more active in the $CELD_{50}$ test than in the LAL test. The pyrogenicity test appears less sensitive but the FI_{40} dose is a large amount of pyrogen.

A significant rank correlation was found between LAL reactivity and mouse lethality ($r = 0.97$ $p < 0.0001$) chick embryo lethality ($r = 0.95$ $p < 0.0002$) and pyrogenicity ($r = 0.91$ $p < 0.0008$) (Fig. 3) The highest correlation was found between mouse lethality and chick embryo lethality ($r = 0.98$, $p < 0.0001$) and the lowest between chick embryo lethality and pyrogenicity ($r = 0.90$ $p < 0.001$) Comparison between the mouse lethality and pyrogenicity also rendered a lower coefficient than between any of the other biological tests correlated ($r = 0.93$ $p < 0.0004$)

DISCUSSION

The results of the present study together with earlier investigations (9 19 20 21) prove that water-soluble endotoxins can be extracted from strains of *Bacteroides* *Fusobacterium* and *Veillonella* However in all test systems measuring endotoxic activity the toxicity of the *Bacteroides* LPS has been characteristically low The reason may be that conventionally extracted and purified *Bacteroides* LPS contain only small amounts of the endotoxin proper or more likely that the chemical structure of the endotoxic principle is quite distinct from that of lipid A, i.e the toxic principle of *Salmonella* LPS

The high activity of the Veillonella endotoxins in all test systems is in agreement with earlier reports by *Mergenhagen et al.* (1961) The endotoxic activity of the *F nucleatum* LPS was comparable to or somewhat lower than that of the highly active LPS from *S enteritidis* S-795 This accords with recent chemical studies which have shown that *Fusobacterium* LPS has a lipid A structure similar to that of LPS prepared from Salmonella (7)

The endotoxic capacity of LPS-Ve 5 and LPS-Ve 9 differed considerably in most assays performed, though they were prepared from strains belonging to the same genus. This was true also for LPS-F1 and LPS Fev 1 It is reasonable to assume that the endotoxic principle is the same in the LPS of Ve 5 and Ve 9 as well as in the LPS of F1 and Fev 1 and that the difference in activity more likely may be due to variation in their solubility

The characteristic symptoms of endotoxic poisoning were observed also when the mice were given very small doses of LPS in mixture with the antimetabolite actinomycin-D The markedly potentiating effect of the antimetabolite on the lethality of LPS to mice was also seen by *Garcia et al.* (1975) who examined LPS isolated from *F necrophorum.* A still higher potentiating effect of actinomycin-D has been reported by *Pieroni et al.* (1970) and *Dowling & Feldman* (1970) testing enterobacterial and meningococcal endotoxins, respectively The discrepancy in results may depend on the usage of different strains of mice or in a variation of the antimetabolite in potency between batches of actinomycin-D A difference in potentiating effect on LPS from aerobic and anaerobic bac

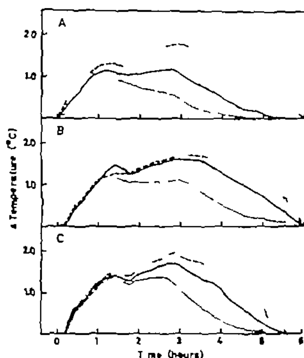


Fig 2 Biphasic pyrogenic response in rabbits to LPS prepared from (A) *B fragilis* strain E 323 (B) *F nucleatum* strain F1 and (C) *V alcalescens* strain Ve 5 Each response curve represents the average of six to eight rabbits. Injection of LPS at time zero.

Doses

- - - (A) 80 0 μg LPS-E 323 (B) 20 0 μg LPS-F1 and (C) 2 0 μg LPS-Ve 5

—— (A) 16 0 μg LPS-E 323 (B) 4 0 μg LPS-F1 and (C) 0.4 μg LPS-Ve 5

(A) 3.2 μg LPS-E 323 (B) 0 8 μg LPS-F1 and (C) 0 08 μg LPS-Ve 5

cording to the designation of the pyrogen test (22) however also the preparation of LPS from NCTC 9343 which was the least potent, meets the requirements for containing pyrogens.

The rabbits responded to all LPS preparations with a biphasic fever (Fig 1) This could be clearly demonstrated with as little as 0 08 μg of LPS-Ve 5 (Fig 2) The first and second peaks of the fever response were recorded at one h and 24 min and three h after inoculation, respectively Where small doses of LPS were inoculated, the temperature usually approached normal six h after administration.

Gelation of Limulus *Lysate*

The capacity of the LPS to gel the *Limulus* lysate varied among the preparations (Table 4) When compared to LPS-S-795 the LPS preparations from the *Veillonella* and the *Fusobacterium* strains were especially active. If only the soft transformation of the lysate was used for the determination of the positive end points, still two to four more higher two-fold dilutions of the LPS preparations were detectable by the LAL. The gelation activity on the *Limulus* lysate of LPS-Ve 5 and LPS-F1 was found equal both when the solid as well as the soft gel were used as criteria of their activity LPS-Fev 1 showed especially pronounced differences between the two stages of lysate transformation as only one tenth of the dose necessary to form a solid gel of the lysate sufficed to make a soft gelation.

Comparisons in sensitivity of the different assays on endotoxins on a weight for weight basis, showed the LAL test to be the most sensitive (Table 5) The amount of endotoxin necessary to produce a positive chick embryo LD_{50}, however was close to that recorded using the *Limulus* lysate test (cf. Table 5) When the criterion of a firm gel adherent to

TABLE 4 *Capacity of LPS Isolated from* Veillonella, Fusobacterium *and* Bacteroides *to Gel the* Limulus *Amoebocyte Lysate (LAL)*

Source of LPS	State of the LAL gel (ng LPS/0 1 ml) Solid	Semi solid b	Rank	Ratio
S-795	7 616	1.523	1	1.0
Ve 5	9 138	2.284	2.5	1 2
Ve 9	24 371	6 855	4	3.2
F1	9 138	2 284	2 5	1.2
Fev 1	36.562	3 426	5	4 8
B 10	585 937	73.242	7	76.9
E 3 3	146 484	30.517	6	19 2
NCTC 9343	781.250	146.690	8	102.6

See Materials and Methods.

b See Materials and Methods.

The solid formation of the gel used for comparison with LPS-S-795 and ranked in order of acthilty

was due to a contaminating pyrogen, for instance traces of bacterial glycopeptides.

No pronounced difference between the reference endotoxin and LPS from the *Veillonella* and *Fusobacterium* strains was observed in the LAL tests. Difficulty in interpreting whether *Limulus* lysate LPS mixture was converted into a solid or semisolid gel at marginal concentrations of endotoxin one h after incubation was experienced. However such concentrations of LPS often showed a solid gel formation when read after two h of incubation. To ensure that positive results are read from marginal concentrations of pyrogen, a further reading at the two h interval would thus be required (cf. Table 4).

Cooper et al (1971 1972) and *van Noordwijk & de Jong* (1976) have found the LAL test to be about ten times more sensitive to pyrogens from aerobic bacteria than the rabbit pyrogen test, which also is in accordance with the results obtained with the *Salmonella* LPS. Endotoxins from the anaerobic bacteria used in this study were detectable in the LAL test at approximately one-hundreth to one-thousandth that dose required for giving a positive pyrogen test (FI_{40}) in rabbits. The *Limulus* lysate gel test is thus the most sensitive test available for the detection of endotoxin from anaerobic microorganisms in their toxic or undegraded state. It is possible that *Limulus* lysate may be relatively more sensitive to LPS from anaerobic bacteria than to LPS from aerobic bacteria. Highly significant rank correlations were found between the results of all tests performed. This indicates that each preparation examined has an endotoxic component that initiates all the biological activities tested.

This study was supported by the NIH Research Grant No DE 03821-01 from the United States Public Health Service.

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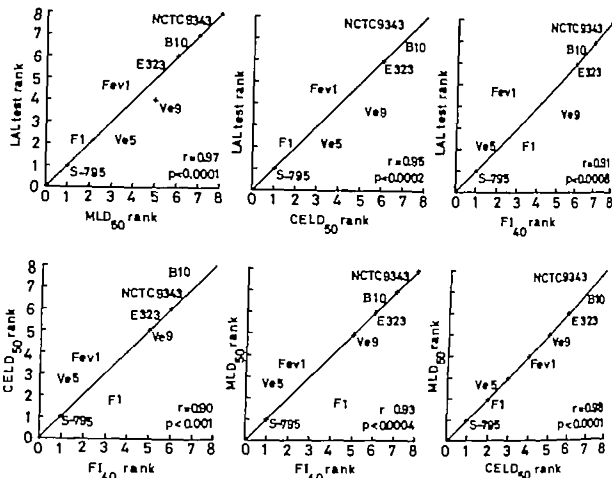


Fig 3 Rank correlation of LPS for LAL activity mouse lethality (MLD_{50}) chick embryo lethality ($CELD_{50}$) and pyrogenicity (FI_{40}) in rabbits The rank of the various LPS is on each axis. The line drawn at 45 degrees represents a perfect correlation ($r = 1.0$) The Spearman rank correlation coefficient is represented by *r* and the significance of this by *p* Abbreviations of LPS used is defined in Table 1

teria may also be considered. After all, the synergistic effect of endotoxin and actinomycin D on mouse lethality is of great value, allowing small quantities of bacterial LPS to be tested for endotoxic activity

Weight by weight the lethal effect of the LPS preparations on eleven-days-old chick embryos ($CELD_{50}$) was considerably higher than in mice (MLD_{50}) Concerning the ranking of the endotoxic activity of the different LPS the two tests were in close agreement ($r = 0.98$ $p < 0.0001$) As compared to the reference endotoxin the lethal effect of the LPS preparations from the *Bacteroides* strains B 10 and NCTC 9343 was comparatively higher in mice than in chick embryos (cf Tables 1 and 2)

As no sonical treatment of the LPS solutions was performed before inoculation of the LPS into chick embryos there may have been an aggregation of the LPS from these two bacterial strains. As reported by *Milner et al* (1963) chick embryos are not very susceptible to aggregated endotoxins. Nevertheless the chick embryo also proved to be useful and versatile for testing of endotoxin from anaerobic bacteria

The pyrexial response of the rabbits to the LPS preparations was biphasic with temperature maxima at one h and 24 min and three h after administration of LPS Again the activity of the *Bacteroides* LPS was characteristically low It may be questioned if the fever response to the NCTC 9343 LPS preparation

Acta path. microbiol. scand. Sect. B, 85: 397-402, 1977

INFECTION OF *CLETHRIONOMYS G. GLAREOLUS SCHREB* (RED MICE) WITH *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS* INJECTED INTRAPERITONEALLY

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Jespersen, A. Infection of *Clethrionomys g. glareolus Schreb.* (red mice) with *Mycobacterium tuberculosis* and *Mycobacterium bovis* injected intraperitoneally. Acta path. microbiol. scand. Sect. B 85: 397-402, 1977.

Groups of mice were injected intraperitoneally with doses from 10^{-} to 10^{-8} mg semi-dried culture of two strains of *M. tuberculosis* or two strains of *M. bovis*. Two mice from each group were killed 1, 1½ months and 3 months after injection; the remaining animals lived until death occurred spontaneously. The number of bacteria in the organs was evaluated by microscopy and in some cases also by culture. The red mice were strongly resistant to *M. tuberculosis*. A dose of 1.2 million viable units was only able to provoke fatal tuberculosis in a few animals in the group. There was no sign of progressive infection among the animals injected with approximately 10^{4} viable units and lower doses, except for a single animal. The only macroscopical findings were small tubercles in the omentum and lungs and slight enlargement of the spleen and lymph glands. The number of bacteria in the organs was small, except in the few cases where the infection became progressive. The red mice were extremely sensitive to *M. bovis*. Probably one single tubercle bacillus or at any rate, very few bacteria were sufficient to provoke progressive tuberculosis with rapid fatal outcome. Macroscopical lesions, which were often caseous developed consistently in omentum, lymph glands, liver, spleen and lungs, and in rare instances also in peritoneum, kidneys, suprarenal glands, heart, columna and joints. The number of bacteria in the organs was very large and in caseous lymph glands and caseous tubercles in lungs and spleen there were tremendous numbers.

Key words: *Mycobacterium tuberculosis*; *Mycobacterium bovis*; intraperitoneal infection; species differentiation; *Clethrionomys g. glareolus Schreb.* (red mice).

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Received 9 77 Accepted 9 77

In a previous work (*Jespersen* 1977) it was shown that red mice infected subcutaneously are 10^{7} to 10^{8} times more susceptible to *M. bovis* than to *M. tuberculosis*. In the present and two subsequent works, the course of the infection has been examined in red mice after intraperitoneal or intravenous injections of varying doses of the two species.

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RESULTS

M tuberculosis

Table I shows the survival times, tuberculous index and bacterial index for killed and spontaneously dead animals injected with varying doses of the two strains of *M tuberculosis*.

Even though the number of viable units in the suspensions of E 10883 H was somewhat lower than in the suspensions of V 11381 H, the effect of the first named strain was the stronger. All the animals in the group injected with 10^{-4} mg of E 10883 H became infected, whereas none in the group injected with the corresponding dose of V 11381 H showed any signs of tuberculosis. The tendency was the same in the other groups.

In the groups injected with 10^{-1} mg (1–2 million viable units) two mice died after 76 and 83 days, both of tuberculosis in the lungs, and in five other animals the disease was slightly progressive. The remaining five animals showed no sign of progressive infection.

In the 10^{-2} group, two animals had slight and one medium progressive tuberculosis. The animal which died on day 161 had no tubercles in the lungs.

Except for one mouse, the animals in the group injected with 10^{-3} mg or lower doses showed no signs of progressive infection.

The macroscopical lesions were small in all the groups. In the 10^{-} group these were limited to small tubercles in the omentum and lungs and slight enlargement of the spleen and lymph glands. There were no tubercles in liver spleen or kidneys, and the lymph glands were not caseous. This was also the case in the animals that died of the infection. The number of bacteria in the organs was moderate in seven of the 12 animals the index was ≦ 1

In the groups 10^{-3} to 10^{-} there were some animals with a few small tubercles in the lungs and slight enlargement of the lymph glands. The processes showed no definite tendency to diminish during the experimental period. The organs contained only few bacteria, the index being ≦ 1 in all animals. The bacteria could often be demonstrated by

...ice Injected Intraperitoneally with Varying Doses (mg, Viable U its) of Two Strains of M tuberculosis

...e	Tub. index	Bact. index	Surv. time	Tub index	Bact. index	Surv time	Tub. index	Bact. index
...umber of viable units								
10^{-4}	17 × 10		10^{-4}	1 7		10^{-4}	0.2	
33	0	0	k 53	0	0	k 53	0	0
33	1	0 5	k 93	0	0	k 53	0	0
95	1	0.3	k 95	0	0	k 96	0	0
95	1	0 5	+ 332	0	0	k 96	0	0
273	1	0 3	+ 394	0	0	+ 241	0	0
128	1	0				+ 242	0	0
...umber of viable units								
10^{-4}	13 × 10		10^{-}	1.3				
31	1	0 3	k 31	1	0 5			
32	1	1.0	k 34	1	0.5			
96	1	0 3	k 97	1	0.3			
97	1	0.3	k 97	1	0 3			
147	0	0	+ 134	1	0			
198	1	0 3	+ 207	1	0.3			

MATERIAL AND METHODS

Experimental Groups of mice, each consisting of six animals, were injected intraperitoneally with doses varying from 10^{-1} to 10^{-8} mg semidried culture of *M tuberculosis* strains V 11381 H and E 10883 H and *M bovis* strains T 3474 B and T 3605 B. The bacterial suspensions of strains V 11381 H and T 3474 B were the same as those used in examination of the subcutaneous infection (*Jespersen* 1977) Two mice from each group were killed 1–1½ and 3 months after injection the remaining animals lived until death occurred spontaneously At autopsy smears were made from liver spleen lungs and lymph glands and were stained by the Ziehl Neelsen method In animals where no macroscopical tuberculous lesions were found, or where lesions were small or uncharacteristic, culture was made from lymph glands, spleen and lungs. Before inoculation on to four Löwenstein-Jensen tubes, the organ suspension was treated with 4 per cent natron.

Experimental animals Red mice aged 2–5 months, bred at the farm belonging to Statens Seruminstitut, were distributed at random into earthenware jars with one animal in each. Each experimental group consisted of equal numbers of females and males.

Bacterial strains M tuberculosis strain V 11381 H and *M bovis* strain T 3474 B were freshly isolated. *M tuberculosis* strain E 10883 H and *M bovis* strain T 3605 B were approximately 3-year old laboratory strains, the virulence of which had been maintained by inoculation on to Löwenstein-Jensen medium alternating with animal passages. Virulence determination showed that all strains were strongly virulent for guinea pigs. The *M bovis* strains were strongly virulent and the *M tuberculosis* strains weakly virulent for rabbits. There was no difference in the virulence of the freshly isolated and the laboratory strains.

Bacterial suspension for infection The strains were grown in Besredka fluid medium and the culture was about 10 days old when used. The preparation of bacterial suspensions for infection of the animals has been described in detail elsewhere (*Jespersen* 1977) Suitable dilutions of each suspension inoculated on to ten tubes of Löwenstein Jensen medium showed that 1 mg of culture of V 11381 H, E 10883 H, T 3474 B and T 3605 B contained 17×10^4 13×10^4 69×10^4 and 8×10^4 viable units, respectively

Injection of animals The mice were injected intraperitoneally with 0.2 ml of the various dilutions.

Registration The degree of tuberculosis in the individual animals and the number of bacteria in the organs were indicated by means of an index from 0–5 based on macroscopical and microscopical findings and results of culture (*Jespersen* 1977) This applies both to the killed and the spontaneously dead animals.

TABLE 1 *Survival Times (Days) Tuberculosis Index and Bacterial Index for Killed and Spontaneously De[ad]*

Surv time	Tub. index	Bact. index	Surv time	Tub. index	Bact. index	Surv time	Tub. index	Bact. index
						Strain V 11381 H do[se]		
10^{-1}	17×10^4					10^{-3}	17×10^3	
k 53	3	1.3				k 53	1	0.8
k 53	3	2.3				k 53	1	0.3
k 94	2	0 8				k 95	1	0.5
k 94	2	0 8				k 95	1	0.3
+ 170	2	0 8				k 137	1	0 3
+ 342	1	0.3				+ 200	1	0.3
						Strain E 10883 H do[se]		
10^{-1}	13×10^4		10^{-2}	13×10^4		10^{-3}	13×10^3	
k 31	3	0.3	k 31	1	0.5	k 31	1	0.5
k 32	3	1 0	k 32	3	1.0	k 32	3	1.0
+ 76	5	3 8	k 96	3	0.5	k 96	2	0.5
+ 83	5	1.8	k 97	4	1 0	k 97	1	0 3
+ 116	2	0.5	+ 161	[illegible]	0 5	+ 137	0	0
+ 164	3	1.3	k 363	1	0.5	k 363	1	0.3

k = killed + = spontaneously dead.

d Mice Injected Intraperitoneally with Varying Doses (mg, Viable Units) of Two Strains of M. bovis

s	Tub. index	Bact. index	Surv time	Tub. index	Bact. index	Surv time	Tub. index	Bact. index
mber of viable units								
	[illegible] 69			10^{-3} 7			10^{-4} 0.7	
[illegible]	5	5.0	+ 47	4	3.5	+ 50	5	4.8
[illegible]	4	1.0	k 53	3	1.7	k 52	0	0
[illegible]	5	5.0	+ 56	5	5.0	+ 54	5	3.5
[illegible]	5	5.0	+ 81	5	5.0	+ 86	5	3.5
[illegible]	5	5.0	+ 101	5	4.8	+ 91	5	5.0
[illegible]	5	3.4	+ 129	5	3.8	+ 230	5	4.4
mber of viable units								
				10– 0.8			10^{-4} 0.1	
			k 32	1	0.2	k 32	0	0
			k 34	0	0	k 34	0	0
			+ 83	4	2.0	k 96	5	4.2
			k 96	3	1.0	k 97	0	0
			k 97	0	0	+ 100	0	0
			+ 137	3	0.4	k 363	0	0

tain or contained gray or yellow tubercles up to the size of hempseeds. The lymph glands—particularly the porta, mesenterial and tracheal glands—were the size of nut kernels and completely yellow. The spleen was tremendously enlarged and contained round, yellow tubercles, or caseous tubercles of more irregular form located particularly at the edge or at the poles, or caseous masses forming a belt-like section across the spleen. In extreme cases, the spleen was transformed into a uniform, solid, whitish-yellow mass. Where the course of infection was very protracted, the spleen of some of the animals contained round, thin-walled abscesses up to the size of cherries, with a content of thin, turbid fluid. There were a few cases where the spleen consisted entirely of a conglomerate of these fluid-filled abscesses and where there was apparently no normal tissue remaining. The liver contained gray or yellow tubercles. There were hempseed-sized or pea-sized yellow tubercles in the lungs, or a lung lobe or the whole lung was transformed into a uniform, whitish-yellow caseonecrotic mass.

In addition to the findings in the organs named, there were sometimes macroscopical lesions in the peritoneum (particularly in the capsule of the kidneys) in the kidneys, suprarenal glands, heart, columna and joints, as well as in all the lymph glands in the abdomen and thorax, and in the peripheral lymph glands in the axilla, inguen and neck.

The number of bacteria in the organs was very large in the caseous lymph glands and in the caseous tubercles in lungs and spleen it was enormous. Smears from these organs resembled pure cultures of tubercle bacilli.

Recovery was seen in rare cases in the liver where it could be complete, and in the spleen, where it was only partial.

DISCUSSION

After intraperitoneal injection, some tubercle bacilli will be retained by the omentum, while others will spread rapidly through the lymphatic vessels and via the ductus thoracicus to the venous system. When doses of 10 mg culture are injected, *M. bovis* can be demonstrated in the blood after one minute and *M. tuberculosis* after 8 minutes (*Jespersen* 1975). With doses of 10^{-4} and 10^{-6} mg cul-

TABLE 2 *Survival Times (Days) Tuberculosis Index and Bacterial Index for Killed and Spontaneously D*

Surv time	Tub. index	Bact. Index	Surv time	Tub. index	Bact. Index	Surv time	Tub. index	Bact. index
						Strain T 3474 B do		
10^{-1}	69×10^{5}		10^{-3} : 69×10^{3}			10^{-5}	69×10	
+ 6	1	0	+ 29	5	5 0	+ 49	5	2.8
+ 6	1	0	+ 34	5	5 0	+ 51	5	4.3
+ 18	4	4 8	+ 38	5	5 0	+ 64	5	2.4
+ 18	5	5 0	+ 47	5	5 0	+ 68	5	5.0
+ 21	5	5 0	+ 48	5	5 0	+ 68	5	5 0
+ 21	5	5 0	+ 59	5	5 0	+ 77	5	5 0
						Strain T 5605 B		
10^{-1}	8×10^{5}		10^{-3}	8×10^{3}		10^{-5}	8×10	
+ 19	5	4.8	k 31	4	2.5	k 31	4	1.9
+ 20	5	5 0	k 32	4	1 9	k 32	3	0.7
+ 20	5	5 0	+ 54	5	3 8	+ 59	5	4.5
+ 24	5	3.5	+ 59	5	4 8	k 96	5	3.8
+ 29	5	4 7	+ 65	5	3.9	+ 98	5	4.8
+ 41	5	4 8	+106	5	3 2	+127	4	2.5

k = killed + = spontaneously dead.

culture but only exceptionally by microscopy and then only in the lymph glands Culture from lymph glands and lungs showed a marked difference in the number of viable units during the experimental period. They could finally disappear completely from the lungs but in the lymph glands they were still viable for a very long time (up to one year)

M bovis

Table 2 shows the survival times, the tuberculosis index and the bacterial index for the killed and spontaneously dead animals injected with varying doses of the two strains of *M bovis* Taking into consideration that the number of viable units in the bacterial suspensions of T 3474 B was considerably larger than in the corresponding suspensions of T 5605 B the two strains seemed to have had the same effect.

The red mice were extremely sensitive to *M bovis* Doses of down to 7 viable units provoked progressive and rapidly fatal tuberculosis in all the animals. Only one of the mice injected with 0 7 viable units of T 3474 B and two in the group injected with 0 8 viable units of T 5605 B showed no signs of infection. The survival times for the animals injected with the three largest doses were prolonged the lower the dose while they were independent of dosage in the animals given the three smallest doses.

The macroscopical lesions in the organs in the 10^{-1} group were considerable, despite the short survival times of the animals. There were gray or yellow tubercles in the spleen and lungs, and the lymph glands in the abdomen and thorax were very enlarged and partially or completely caseous. The bacterial index was not below 3.5 and was maximal for half the animals.

In the mice in the 10^{-3} and 10^{-4} groups which were killed a month after injection the autopsy findings were less extensive, but microscopy revealed that the infection was in full progress. The tubercles developed later in the lungs than in the spleen. The tuberculous lesions were much more developed in the second month than in the first, and in the third month they had reached their maximum. The omentum was retracted and enlarged and consisted of a uniform yellow

Ind Mice Injected I traperitoneally with Varying Doses (mg, Viable Units) of Tw Strains of M bovis

Surv time	Tub index	Bact. index	Surv time	Tub. index	Bact. index	Surv time	T b. index	Bact. index
number of viable units								
10^{-4} 69			10^{-4} 7			10^{-4} : 0.7		
+ 47	5	5.0	+ 47	4	5.5	+ 50	5	4.8
[illegible] 55	4	1 0	k 53	5	1 7	k 52	0	0
+ 66	5	5.0	+ 56	5	5.0	+ 54	5	3.5
+ 62	5	5.0	+ 81	5	5.0	+ 86	5	3.5
+ 82	5	5.0	+ 101	5	4.8	+ 91	5	5.0
+153	5	3 4	+ 129	5	5.8	+ 230	5	4 4
number of viable units								
			10- 0.8			10^{-4} 0.1		
			k 32	1	0 2	k 52	0	0
			k 54	0	0	k 54	0	0
			+ 83	4	2.0	k 96	5	4.2
			k 96	3	1.0	k 97	0	0
			k 97	0	0	+ 100	0	0
			+ 137	3	0 4	k 363	0	0

mass or contained gray or yellow tubercles up to the size of hempseeds. The lymph glands —particularly the porta, mesenterial and tracheal glands—were the size of nut kernels and completely yellow The spleen was tremendously enlarged and contained round, yellow tubercles, or caseous tubercles of more irregular form located particularly at the edge or at the poles, or caseous masses forming a belt-like section across the spleen. In extreme cases, the spleen was transformed into a uniform, solid, whitish-yellow mass Where the course of infection was very protracted, the spleen of some of the animals contained round, thin-walled abscesses up to the size of cherries, with a content of thin turbid fluid. There were a few cases where the spleen consisted entirely of a conglomerate of these fluid-filled abscesses and where there was apparently no normal tissue remaining. The liver contained gray or yellow tubercles. There were hempseed-sized or pea-sized yellow tubercles in the lungs, or a lung lobe or the whole lung was transformed into a uniform, whitish-yellow caseonecrotic mass.

In addition to the findings in the organs named, there were sometimes macroscopical lesions in the peritoneum (particularly in the capsule of the kidneys) in the kidneys, supra renal glands, heart, columna and joints, as well as in all the lymph glands in the abdomen and thorax, and in the peripheral lymph glands in the axilla, inguen and neck.

The number of bacteria in the organs was very large In the caseous lymph glands and in the caseous tubercles in lungs and spleen it was enormous. Smears from these organs resembled pure cultures of tubercle bacilli.

Recovery was seen in rare cases in the liver where it could be complete, and in the spleen, where it was only partial.

DISCUSSION

After intraperitoneal injection, some tubercle bacilli will be retained by the omentum, while others will spread rapidly through the lymphatic vessels and via the ductus thoracicus to the venous system. When doses of 10 mg culture are injected, *M bovis* can be demonstrated in the blood after one minute and *M tuberculosis* after 8 minutes (*Jespersen* 1975) With doses of 10- and 10^{-4} mg cul.

ture both species can be found in the lungs after 5 and 60 minutes, respectively but *M bovis* is present in the largest number (*Jespersen* unpublished)

Characteristic for infection with *M tuberculosis* is that multiplication of the bacteria is limited and ceases early in the organs, except in the lymph glands where it continues for a long time that macroscopical lesions develop in a few organs only that the lesions are small and generally do not end in caseous necrosis but often heal especially where smaller doses are injected. In contrast, *M bovis* multiplies strongly in the red mice and multiplication continues until the animals die. In addition to the universal extent of the *M bovis* infection, the macroscopical lesions are very large they achieve maximum development in animals injected with a few bacilli especially in cases with protracted survival times finally the lesions end in extensive necrosis which transforms the tissue into caseous masses healing seldom takes place

A previous work (*Jespersen* 1954) deals with pilot studies on red mice, using varying doses of *M tuberculosis* and *M bovis* These experiments have been reproduced and confirmed by *Hauduroy* & *Buse* (1957) and *Buse* (1961) In agreement with the writer those authors conclude that red mice are suitable for species determination. *Jespersen* recommends a test dose in the range of 10^{-3} to 10^{-5} mg and *Buse* a dose of 10^{-4} mg

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INFECTION OF *CLETHRIONOMYS G GLAREOLUS SCHREB* (RED MICE) WITH *MYCOBACTERIUM TUBERCULOSIS* INJECTED INTRAVENOUSLY

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Jespersen, A., Bentzon, M W & Møller S. Infection of *Clethrionomys g glareolus Schreb* (red mice) with *Mycobacterium tuberculosis* injected intravenously Acta path. microbiol. scand. Sect B, 85 403–414 1977

Groups of red mice were injected intravenously with doses varying from 11 viable units to about 10^{8} viable units of a virulent strain of *M tuberculosis* grown in Dubos Tween medium and dispersed by means of ultrasonics The course of the infection was determined by quantitative culture from the organs of animals killed at various times after the injection and by the survival times of animals that died spontaneously Quantitative culture showed that the tubercle bacilli multiplied strongly in the organs during the first 2 3 weeks after the injection. Multiplication then ceased completely in liver spleen and lungs In the lymph glands the bacilli continued to multiply but at a very slow rate. After the period of initial multiplication, the number of bacteria in liver and spleen decreased gradually during the whole experimental period (1 year) although it varied considerably from animal to animal after the second month. In the lungs, the number remained almost constant in the animals injected with large doses, but in those given doses $\leq 10^{4}$ viable units, it became reduced from the second month, and in some cases the bacteria disappeared completely Total elimination of the bacteria was also observed in the liver and spleen of a few animals but never in the lymph glands. The mean survival times of animals injected with the four largest doses were prolonged from 81 to 310 days the smaller the dose. In the animals injected with doses $\leq 10^{4}$ viable units, the mean survival times, which were independent of dosage, were not significantly different and not significantly less than those of a group of non-infected animals. The macroscopical tuberculous lesions were small in all groups. Enlargement of the spleen, slightly enlarged lymph glands and possibly tubercles in the lungs were the most frequent findings. The number of bacteria in the organs was large in all animals injected with the large doses, but only in about half of those given small doses.

Key words *Mycobacterium tuberculosis;* intravenous infection *Clethrionomys g glareolus Schreb* (red mice)

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Received 14.vii.77 Accepted 14.vii.77

In recently published works (*Jespersen* 1977 a, b), comprising small groups of animals an approximate picture was obtained of the course of infection in red mice injected subcutaneously or intraperitoneally with varying doses of *M. tuberculosis* or *M. bovis*. Red mice proved to be resistant to *M. tuberculosis* and most resistant after subcutaneous injection where the smallest dose that could provoke progressive infection was 10^7 to 10^8 viable units. On the other hand these animals were extremely susceptible to *M. bovis*: very few viable units, or probably only a single bacterium, provoked a fatal infection whether injected intraperitoneally or subcutaneously.

The present study reports the course of the infection in red mice injected intravenously with different doses of a virulent strain of *M. tuberculosis*. Based partly on the survival times and partly on quantitative culture from the organs at various times after the injection a detailed picture of the course has been obtained. A subsequent paper reports similarly the course of infection with a strain of *M. bovis*.

MATERIAL AND METHODS

Experimental

Experiment a. Quantitative culture from organs at different times after injection with varying doses. Groups of red mice were injected intravenously with dilutions 10^0, 10^{-2}, 10^{-4} and 10^{-6} of a strain of *M. tuberculosis* grown in Dubos fluid Tween medium and dispersed by means of ultrasonics. Twenty four hours after the injection and then every fourth to fifth day during a period of up to day 126 (for the 10^0 group day 101) one animal from each group was killed and organs for quantitative culture were removed at sterile autopsy. The examination was supplemented by quantitative culture from mice killed in the period day 196 to day 337. For this purpose, six animals were selected at random from the groups injected with dilutions 10^{-2} to 10^{-6} in experiment b.

Experiment b. Survival times of animals injected with various doses. Groups of 20 red mice were injected intravenously with dilutions 10^0 to 10^{-7} of the same bacterial suspensions as used in experiment a. As control of the capability of the animals to survive a group of 20 non-infected animals was included. Except for the four × six mice which, as mentioned above, were used for quantitative culture, the animals were allowed to live until death occurred spontaneously. The experiment was concluded 2 years after the inoculation. Smears for microscopy were made from lymph glands, liver, spleen and lungs. In animals where no definite macroscopical signs of tuberculosis were found, culture was also carried out from the same organs. Before inoculation on to four Löwenstein Jensen tubes, the organ suspension was treated with 4 per cent natron. In the case of nine mice that were killed at the end of the experiment, the suspension was inoculated both untreated and after treatment with natron.

Experimental Animals

The red mice, which were bred at the farm belonging to Statens Seruminstitut, were 2–5 months old at the start of the experiments. The mice were distributed at random into earthenware jars with one animal in each. Each experimental group consisted of equal numbers of females and males.

Infection

A strain of *M. tuberculosis* E 36499 H, freshly isolated from sputum, was grown in Dubos fluid Tween medium and subcultured three times. About 20 ml culture was shaken until homogeneous macroscopically and then exposed to ultrasonics for 10 minutes. From that suspension ten-fold dilutions down to 10^{-7} were made with diluted Sauton (one part Sauton medium and three parts distilled water). Inoculation of 0.1 ml of three suitable dilutions on to ten Löwenstein Jensen tubes from each dilution showed that 0.2 ml 10^{-7}, i.e. the lowest dose per mouse, contained 11 viable units.

The mice were injected with 0.2 ml bacterial suspension into a tail vein. The lowest dose was injected first, then the ten times higher dose and so on. The animals were injected in random order within the individual groups.

Quantitative Culture

Culture was performed from the left liver lobe, the whole spleen, the whole left lung and the pooled mesenterial and lumbar lymph glands. The organs were comminuted in mortar and 2 ml diluted Sauton medium was added dropwise. From this suspension (10^0) suspensions 10^{-1}, 10^{-2} etc. were made with diluted Sauton medium, and 0.1 ml of three suitable dilutions were inoculated on to each of four Löwenstein Jensen tubes. The inoculated tubes were placed in horizontal position for 24 hours and then incubated at 37 °C. The colonies were counted when they had reached a suitable size.

Registration

For the animals selected for determination of the survival times, the degree of tuberculosis and the number of bacteria in the organs were recorded by means of an Index based on macroscopical and microscopical findings and the results of culture.

Tuberculosis Index

0 organs normal, culture negative
1 no definite specific lesions, culture positive
2 small tuberculous lesions, mainly regressive
3 = slight progressive tuberculosis
4 = medium progressive tuberculosis
5 severe progressive tuberculosis

Bacterial Index

0 = no bacteria
1 microscopy negative, culture positive
2 = 1 20 bacteria by microscopy (fluorescence)
3 = 21 99 bacteria
4 = 5-50 bacteria per sight field
5 = >50 bacteria per sight field

Virulence Determination of H1 and E 36499 H

Three groups of two guinea pigs were injected intraperitoneally with 1 ml of dilutions 10^{-4} 10^{-} and 10^{-6} of the same suspensions as those used for injection of the mice. The results (Table 1) show that the strain was highly virulent for guinea pig

TABLE 1 *Virulence Determination of Strain of M. tuberculosis on Guinea Pigs*

Viable units (i. p.)	Survival time (days)	Degree of tuberculosis
6×10^{6}	72	5)
6×10^{6}	72	5
6×10^{5}	113	5
6×10^{5}	121	5
6×10^{3}	128	5
6 10^{6}	169	5

) 5 = severe generalized tuberculosis

Statistical Methods

Quantitative culture A special method was used for estimation of the number of viable units in the different organs The number of colonies observed in the dilution series did not correspond to dilution factor 10 A correction of the colony count was carried out in accordance with the model used by Engbæk et al (1967) $u = \frac{\lambda}{1 + \lambda}$ where λ is the true number of bacterial units. Usually two consecutive dilutions giving colony counts between 100 and 5 could be used for estimation of the number of bacterial units in the undiluted suspension. The estimation method is based on the maximum likelihood principle. Details of the method will be given in subsequent paper Only one animal was taken out each time for culture from the organs, and thus a direct estimate of the random variation from animal to animal cannot be made. Since corresponding animals injected with the three lowest doses were examined on the same days, the random variation can be estimated by the residual variance in a two-way analysis of variance provided the curves run parallel. Such analyses have been made for two periods, i.e. days 17 62 and days 66–126, for each organ separately It was examined as to whether there were significant differences between doses and between times within the period.

Survival times. Pairwise comparisons between the survival time distributions for doses 10^{-7} to 10^{6} were carried out by Haldane two sample tests (1945) P values < 5 per cent are considered significant and are shown in brackets in the text.

RESULTS

Quantitative Culture from Organs

Injection dose 10^{6} 11×10^{6} viable units (Fig 1) In this and the following three figures the logarithm of the number of viable units in lymph glands, liver spleen and lungs is plotted on the ordinate and days after injection on the abscissa.

Tubercle bacilli could be found in all organs after 24 hours The number was greatest in the liver and decreased via spleen and lungs to the lymph glands. Except in the liver the bacteria multiplied until day 9 to day 13, most strongly in the lymph glands. In liver spleen and lungs, the number of viable units then remained at about the same level, but varied considerable from animal to animal after the second month. In contrast, multiplication continued in the lymph glands during the remaining experimental period, but at a much slower rate The curve for the glands, which was below the curve for the lungs until day 38, then crossed the latter and thereafter remained constantly higher

Injection doses 10^{-4} 10^{-3} and 10^{-2} 11×10^{3} 11×10^{2} and 11×10 viable units (Figs 2 3

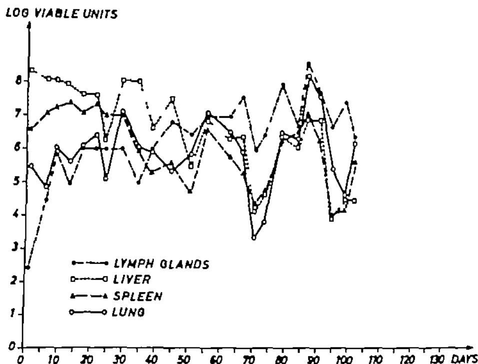


Fig 1 Dose 10^0 11×10^7 viable units.

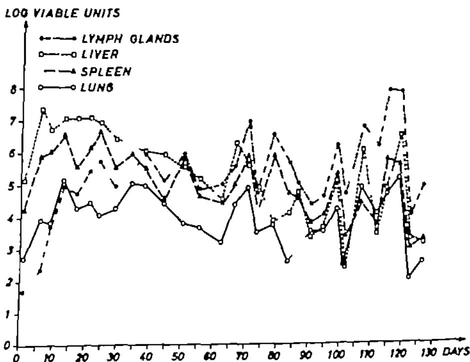


Fig 2 Dose 10^{-2} 11×10^5 viable units.

Figs 1–4 Number of viable units in organs of red mice injected intravenously with doses from 10^0 to 10^{-6} *M tuberculosis* and killed at various times after injection. *Ordinate* log viable units *Abscissa* days after injection.

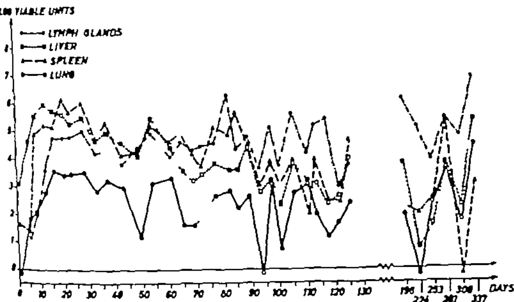


Fig 3 Dose 10 11 × 10³ iable units.

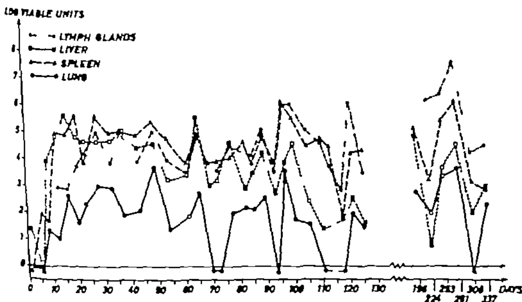


Fig 4 Dose 10 11 × 10 iable units.

4) Throughout the whole experimental period, the number of viable units in all the organs of the animals in the 10⁻³ group (Fig 2) was higher than the corresponding values in Fig. 3 which in turn were higher than those in Fig 4 However apart from these differences in level, the curves had generally the same course. In the groups where tubercle bacilli could be demonstrated after 24 hours, the distribution in the individual organs was

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as in the 10^{6} group *i.e.* the number was greatest in the liver and decreased via the spleen and lungs to the lymph glands. The bacteria multiplied strongly in all organs for 2 to 3 weeks, after which multiplication ceased in liver spleen and lungs, and was strongly inhibited in the lymph glands. The curves for liver and spleen which were highest and ran parallel, had a slightly falling tendency during the remaining experimental period. The curve for the lymph glands was below the curves for liver and spleen to begin with, but crossed them during the second to third month and then lay above them, as was also evident in the late period (day 196 to day 337). The curve for the lungs was the lowest, as was evident in the 10^{-4} group and particularly in the 10^{-6} group. It had a falling tendency after day 40 not only was the number of viable units small in many of the animals, but culture was negative in a few cases, *viz.* two animals in the 10^{-4} group and five animals in the 10^{-6} group.

After the initial multiplication had ceased and up to the end of the second month, the number of viable units in the individual organs varied remarkably little from animal to animal while it varied considerably in the individual animals after the second month.

Statistical analysis. The statistical evaluation of the curves for the three doses is based on two-way analysis of variance carried out for each organ in the periods days 17–62 and days 66–126 (periods 2 and 3). The variation between the animals is greater in period 3 than in period 2. The ratio between the variance estimates is 4.7 5.4 2.8 and 2.8 for lymph glands, liver spleen and lungs, respectively. The ratios are all significantly larger than 1 (P values < 0.05 < 0.05 2.5 and 2.5 per cent).

It applies to all organs and to both periods that the number of bacteria decreases the lower the dose the differences being significant for lymph glands, liver and lungs. There is significant dependence on time for liver and spleen in period 2 but not in the other analyses. The values for liver and spleen are markedly lower in period 3 than in period 2 ($P < 0.05$ per cent). The same tendency can be seen for the lungs, while the tendency is increasing for the lymph glands (not significant $P > 10$ per cent).

Survival Times

Table 2 shows the survival times in days for red mice injected with doses from 11 viable units to 11×10^{7} viable units. The tuberculosis index and the bacterial index for the individual animals are also shown. Fig 5 shows the survival times in graphical form, with the percentage number of animals still alive at the various times plotted on the ordinate and days after injection on the abscissa.

M. tuberculosis provoked a slowly developing infection in the red mice which sometimes could be progressive. The number of mice with tuberculosis index 3–5 *i.e.* cases where the tuberculosis was considered to be progressive varied in the groups injected with the four smallest doses from 36 to 79 per cent, and in the groups injected with the four largest doses from 85 to 100 per cent. In seven mice injected with the smaller doses, it was not possible to demonstrate tubercle bacilli by culture. However in all of eight mice killed on day 736 to day 745 where, in contrast to what was the case with the seven mice culture was carried out without natron treatment, tubercle bacilli were found in one or more organs.

The course of the disease was markedly chronic and independent of dosage in the animals injected with the four smallest doses. The mean survival times, which varied from 456 to 540 days, are not significantly different, nor are they significantly different from the mean survival time of the non infected animals (608 days). It is noteworthy that the curve for the latter group and the curve for the animals injected with 10^{-6} ran parallel up to day 520 after which a number of deaths occurred in the 10^{-6} group and none in the control group. In the animals injected with the four largest doses, the survival times decreased significantly the higher the dose from 310 to 81 days ($10^{\ }$ ~ 10^{-3} $P = 3.5$ per cent

TABLE 2 *Sur val Times Tuberculous and Bacterial Indices | Spontaneously Dead and Killed R d Mice I jected I t avenously with Varying Doses (Dilution I iable Units) of Strain of M tuberculosis F gures Below the Table R present the Median Sur val Tim s, th Percentage N mber of Animals with Progressive Tuberculosis (I de 3–5) and the A erage Bacterial Ind*

	10^{-} 11			10^{-4} 11 × 10			10^{-4} 11 × 10^{8}			10^{-4} 11 × 10^{7}		
Non-infected controls	Surv time (days)	Degree of tub	Bacterial index	Surv time (days)	Degree of tub	Bacterial index	Surv time (days)	Degree of tub	Bacterial index	Surv time (days)	Degree of tub.	Bacterial Index
165	83	1	0.5	240	1	1.5	260	2	1.8	43	5	3.5
240	141	1	0.3	287	2	2.0	275	0	0	189	5	4.8
263	163	1	0.5	370	4	4.3	283	0	0	208	4	3.5
367	218	1	1.3	379	1	1.0	312	5	5.0	269	5	4.8
398	220	4	3.5	474	3	1.8	334	0	0	294	2	1.8
415	274	4	4.3	480	4	4.5	378	4	4.8	404	5	5.0
438	276	5	4.8	507	5	4.8	450	2	1.3	535	4	4.0
495	297	5	5.0	573	5	4.8	493	4	4.3	537	1	0.3
514	317	4	4.5	592	2	2.8	521	1	2.3	557	5	4.8
520	445	1	0.3	597	0	0	527	0	0	590	5	5.0
696	467	0	0	626	4	4.3	547	5	4.8	593	5	4.8
703	487	1	1.5	636	5	5.0	604	0	0	632	5	5.0
716	584	5	5.0	677	5	5.0	657	5	5.0	k 745	3	1.3
k 745	679	2	0.8	k 742	1	0.5	674	1	0.3	k 745	1	1.0
k 745	696	3	3.3									
k 745	k 736	4	3.8									
k 745	k 738	2	2.0									
k 745	k 736	2	2.0									
k 745	k 742	3	2.3									
k 745	k 742	1	1.8									
608	456	45	2.4	540	57	3.0	462	36	2.1	536	79	3.5

k = killed.

TABLE 2. (cont.)

10^{-3} 11×10^4			10^{-2} 11×10^5			10^{-1} 11×10^6			10^{0} 11×10^7		
Surv time (days)	Degree of tub.	Bact erial index	Surv time (days)	Degree of tub.	Bact erial index	Surv time (days)	Degree of tub.	Bact erial index	Surv time (days)	Degree of tub.	Bact erial index
87	5	4.8	78	4	4.3	51	4	3 0	32	4	4.3
162	4	4 0	109	5	3.5	66	5	5 0	34	4	3.8
172	5	5.0	120	5	4.8	76	4	4.0	46	5	5 0
229	5	4.8	138	5	4.8	88	4	4.3	50	5	5.0
296	5	4.8	148	2	2.8	94	5	4.8	54	4	3 8
302	5	2.3	148	4	4.8	97	4	4.3	62	5	4.5
304	5	3.3	164	5	2.8	104	5	4.8	73	5	4.3
316	5	4.5	167	5	4 8	119	5	4 8	78	5	4.5
323	5	5.0	169	5	4 8	127	5	5.0	78	5	5.0
463	5	4 8	182	4	4 0	130	4	4.5	80	5	3.8
530	0	0	200	2	2.3	133	5	5 0	82	5	5.0
544	4	4.3	207	5	5.0	134	5	4.8	86	5	3.5
596	1	0.3	280	4	4.3	137	5	4.8	89	4	3 8
k 745	5	1 0	293	5	4.5	143	5	5 0	102	4	3.5
			315	5	4 8	178	5	4.8	105	4	3 8
			322	5	4 8	209	5	5 0	107	5	4.5
			325	3	2.3	239	5	5 0	120	4	4 0
			345	2	2.0	273	5	5.0	124	4	4.3
			366	3	3.8	308	3	3.5	183	5	5 0
			464	5	5 0	350	1	0.5	186	5	4.8
310	86	3.3	191	85	4 0	132	95	4 4	81	100	4.3

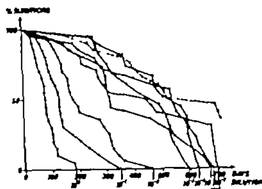


Fig 5 Survival curves of spontaneously dead red mice injected intravenously with doses from 10^{0} to 10^{-} (11×10^{7} to 11 viable units) *M tuberculosis* and group of non-infected control animals (stippled curve) *Ordinate* percentage survivors *abscissa:* days after injection.

$10^{-4} \sim 10^{-1}$ P = 0 8 per cent $10^{-} \sim 10^{0}$ P = 0 3 per cent)

The mean survival time of the animals injected with 10 was significantly lower than that of the non-infected animals. Up to day 310, the survival curve for the 10^{-} group ran alongside the curves for the three smallest doses but was below them for the rest of the course.

TABLE 3 *Frequency (Per cent ge) of Enlarged Lymph Glands Enlarged Spleen and T bercles in Lungs of Groups of Red Mice I jected I travenously with Varying Doses (Dilution Viable Units) of St ain of* M. tuberculosis

Dose		Lymph glands	Liver	Spleen	Lungs
10^{0}	11×10^{7}	100	0	75	65
10	11×10^{6}	100	0	65	10
10	11×10^{5}	95	0	45	5
10	11×10^{4}	79	0	57	7
10	11×10^{3}	100	0	50	21
10	11×10^{2}	64	0	29	21
10	11×10^{1}	79	0	50	21
10	11	75	0	50	10

The autopsy findings were generally small. It was particularly the lungs and lymph glands that were attacked. The liver was always normal and the spleen often slightly enlarged, but only in a few exceptional cases were tubercles observed. Table 3 shows the percentage frequency of tubercles in the lungs, enlarged lymph glands and enlarged spleen in the individual injection groups.

It was only in the 10^{0} group that lung lesions were found frequently A few just visible gray tubercles were seen in ten animals, in two that died on days 124 and 183 there were a few pinhead-sized gray tubercles, and in one animal that died on day 186 there were some confluent gray and yellow tubercles. In the other groups, macroscopical lung lesions were found in 5 to 21 per cent of the animals. Generally the lesions were insignificant, but in three animals that lived 1½ years or more, the lungs contained tubercles in which necrosis was present to varying degrees.

Enlarged spleen and enlarged lymph glands were a more frequent finding than tubercles in the lungs. The frequency which varied between 64 and 100 per cent in the glands and between 29 and 75 per cent in the spleen, increased the larger the dose The lymph glands were most often millet-sized but never larger than the size of hempseeds. Necrosis was seen in 15 animals, eight of which were killed at the end of the experiment. The spleen was slightly enlarged, being up to three times the normal volume Tubercles in the spleen were seen in six animals, four of which lived longer

TABLE 2 (cont.)

10^{-3} 11×10^{4}			10^{-2} 11×10^{5}			10^{-1} 11×10^{6}			10^{0} 11×10^{7}		
Surv time (days)	Degree of tub.	Bact erial index	Surv time (days)	Degree of tub	Bact erial index	Surv time (days)	Degree of tub	Bact erial index	Surv time (days)	Degree of tub	Bact erial index
87	5	4 8	78	4	4.3	51	4	3 0	32	4	4 3
162	4	4 0	109	3	3.5	66	5	5 0	34	4	3 8
172	5	5 0	120	5	4 8	76	4	4 0	46	5	5 0
229	5	4.8	138	5	4.8	88	4	4.3	50	5	5 0
296	5	4.8	148	2	2 8	94	5	4 8	54	4	3 8
302	3	2.3	148	4	4 8	97	4	4.3	62	5	4.5
304	3	3.3	164	3	2.8	104	5	4 8	73	5	4 3
316	5	4.5	167	5	4 8	119	5	4 8	78	5	4.5
323	5	5.0	169	5	4.8	127	5	5 0	78	5	5 0
463	5	4 8	182	4	4 0	130	4	4.5	80	3	3 8
330	0	0	200	2	2.3	133	5	5 0	82	5	5 0
544	4	4.3	207	5	5 0	134	5	4.8	86	3	3 5
596	1	0.3	280	4	4.3	137	5	4 8	89	4	3 8
k 745	3	1 0	293	5	4.5	143	5	5 0	102	4	3.5
			315	5	4.8	178	5	4 8	105	4	3 8
			322	5	4 8	209	5	5.0	107	5	4.5
			325	3	2.3	239	5	5 0	120	4	4 0
			345	2	2.0	273	5	5 0	124	4	4.3
			366	3	3 8	308	3	3.5	183	5	5 0
			464	5	5 0	350	1	0.5	186	5	4.8
310	86	3.5	191	85	4.0	132	95	4 4	81	100	4.3

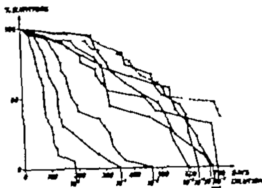


Fig 5. Survival curves of spontaneously dead red mice injected intravenously with doses from 10^{0} to 10^{-7} (11 × 10^{7} to 11 able units) *M. tuberculosis* and group of non-infected control animals (stippled curve) *Ordinate* percentage survivors *abscissa* days after injection.

$10^{-2} \sim 10^{-1}$ P = 0.8 per cent $10^{-1} \sim 10^{0}$ P = 0.3 per cent)

The mean survival time of the animals injected with 10^{-3} was significantly lower than that of the non-infected animals. Up to day 310, the survival curve for the 10 group ran alongside the curves for the three smallest doses but was below them for the rest of the course.

TABLE 3 *Frequency (Percentage) of Enlarged Lymph Glands, Enlarged Spleen and Tubercles in Lungs of Group of Red Mice Injected Intravenously with Varying Doses (Dilution Viable Unit) of Strain of M. tuberculosis*

Dose		Lymph glands	Liver	Spleen	Lungs
10^{0}	11×10^{7}	100	0	75	65
10	11×10^{6}	100	0	65	10
10	11×10^{5}	95	0	45	5
10	11×10^{4}	79	0	57	7
10	11×10^{3}	100	0	50	21
10	11×10^{2}	64	0	29	21
10	11×10^{1}	79	0	50	21
10	11	75	0	30	10

The autopsy findings were generally small. It was particularly the lungs and lymph glands that were attacked. The liver was always normal and the spleen often slightly enlarged, but only in a few exceptional cases were tubercles observed. Table 3 shows the percentage frequency of tubercles in the lungs, enlarged lymph glands and enlarged spleen in the individual injection groups.

It was only in the 10^{0} group that lung lesions were found frequently. A few just visible gray tubercles were seen in ten animals, in two that died on days 124 and 183 there were a few pinhead-sized gray tubercles, and in one animal that died on day 186 there were some confluent gray and yellow tubercles. In the other groups, macroscopical lung lesions were found in 5 to 21 per cent of the animals. Generally the lesions were insignificant, but in three animals that lived 1½ years or more, the lungs contained tubercles in which necrosis was present to varying degrees.

Enlarged spleen and enlarged lymph glands were a more frequent finding than tubercles in the lungs. The frequency which varied between 64 and 100 per cent in the glands and between 29 and 75 per cent in the spleen, increased the larger the dose. The lymph glands were most often millet-sized but never larger than the size of hempseeds. Necrosis was seen in 15 animals, eight of which were killed at the end of the experiment. The spleen was slightly enlarged, being up to three times the normal volume. Tubercles in the spleen were seen in six animals, four of which lived longer

than 1½ years after the injection. For the three smallest doses, there was significant positive correlation between the findings in lymph glands, spleen and lungs (lymph glands ~ spleen P=3 4 per cent spleen ~ lungs P=0 01 per cent)

The number of bacteria in the organs of the individual mice was evaluated by microscopy of smears and recorded as a bacterial index. The means of the indices for lymph glands, liver spleen and lungs in the individual injection groups are shown in Table 4 The number of bacteria was greatest in the lymph glands, and smallest in lungs and liver where the number was almost equal. The number in the spleen lay in between In all organs the number of bacteria increased the larger the dose. This can also be seen from Table 2 where a mean index for the organs is given for the individual mice and below the table a mean index for the mice in the various injection groups.

TABLE 4 *Mean Bacterial Index for Lymph Glands Liver Spleen and Lungs of Groups of Red Mice Injected Intravenously with Varying Doses (Dilution Viable Units) of a Strain of* M. tuberculosis

Dose		Lymph glands	Liver	Spleen	Lungs
10^{0}	11×10^{7}	5 0	4 1	4.2	4 0
10^{-1}	11×10^{6}	4 8	4.3	4.5	4 0
10^{-2}	11×10^{5}	5 0	3 6	4 0	3 4
10^{-3}	11×10^{4}	3 9	3 5	3.5	3 2
10^{-4}	11×10^{3}	4 2	3 1	3 7	3 4
10^{-5}	11×10^{2}	2 7	1 9	1 9	1 9
10^{-6}	11×10^{1}	3 4	2.9	3 2	2 7
10^{-7}	11	3.5	1 9	2.2	2.0

DISCUSSION

Quantitative Culture Method

The ultrasonic-exposed bacterial suspension injected consisted exclusively of single bacteria and very small clumps. After the bacteria are taken up by the cells of the organs, they multiply almost uninhibited, resulting in a new distribution of single bacteria and small and larger clumps. During comminution in mortar the majority of the cells are crushed and the tubercle bacilli are liberated, but the clumps are not separated into single bacteria. After inoculation on to the Löwenstein Jensen medium, both a single bacterium and a clump will grow into one colony and thus the number of colonies will be smaller than the number of bacteria. However as the result of the stronger multiplication, the suspensions of organs from mice infected with *M bovis* will contain more and larger bacterial clumps than those from animals infected with *M tuberculosis* and the difference between the number of bacteria and the number of colonies will be largest for the former suspensions (*Jespersen* unpublished)

Results of Culture

The course of the infection can be divided into three phases. During the first phase which lasts from 2 to 3 weeks, the bacteria multiply almost without any resistance from the organism, resulting in an immunity which has a strong inhibitory effect on the multiplication. In liver spleen and lungs, multiplication ceases completely but continues in the lymph glands, though at a very slow rate. The conclusion of the initial phase is followed by a second phase lasting up to day 45 to day 60 during which the number of bacteria in the organs is almost constant in the individual animals. However during the third phase lasting up to the conclusion of the experiment (day 337) the number of bacteria varies considerably from animal to animal. This reflects the difference in the resistance acquired by the individual mice, thus resulting in progressive infection in some and regressive infection in others.

Taking the second and third phases together it has been shown that the number of bacteria at the beginning of the second phase is largest in liver and spleen, and that it decreases in both organs (most markedly in the liver) during the whole experimental period. Primarily the lymph glands contain fewer but later more bacteria than the liver and spleen, which are by passed during the end of the second phase and the commencement of the

dard. Thus, in the lymph glands multiplication exceeds destruction. The curve for the viable units in the lungs, which in the 10^4 group is generally alongside the curves for liver and spleen, lies just below these in the 10^{-2} and quite separate from them in the 10 and particularly in the 10^{-4} groups. The destruction of bacteria is greater in the lungs than in the liver and spleen, and became complete in a few animals injected with smaller doses.

In experiments with rabbits, whose susceptibility to *M. tuberculosis* and *M. bovis* resembles that of red mice *Lurie* (1928) found by quantitative culture after intravenous injection of a strain of *M. tuberculosis* that the bacteria multiplied in all organs up to the fourth week. Growth then ceased in liver and spleen, but continued undiminished in lungs and kidneys. At the same time a destruction of bacteria commenced, particularly in the spleen. After the second month, the destruction was also apparent in the lungs and growth ceased in the kidneys. Individual differences in resistance were particularly evident after the second month. On essential points, there is thus concordance between the results of *Lurie's* and the present experiment.

Survival Times

Progressive tuberculosis was found in almost all the animals injected with the four largest doses. The mean survival times of the individual groups were more prolonged the lower the doses. Though the organs contained a large number of tubercle bacilli, the autopsy findings were small and limited to tubercles in the lungs and a moderate enlargement of lymph glands and spleen.

In the groups injected with the four lowest doses, the course was markedly chronic. None of the mean survival times was significantly less than that of the non-infected control group. However, 45 per cent of the animals had medium or severe progressive disease, i.e. cases where the cause of death must be presumed to have been tuberculosis. In the majority of the remaining animals, the tubercle bacilli have been present in the organs, particularly in the lymph glands, in considerable numbers throughout the lifetime without apparently causing harm and without having any appreciable influence on the survival times of the mice.

There were seven cases where culture from all organs was negative. However, this is not tantamount to complete bacterial destruction, since before inoculation the organ suspension was treated with natron which can kill up to 90 per cent of the bacteria. Against the supposition of complete destruction is the fact that in eight animals killed at the end of the experiment, where the organs were not treated with natron, all cultures were positive except for two from liver and one from spleen. It must be concluded, therefore, that once they are introduced into the organism of the mouse even in minimal numbers, tubercle bacilli cannot become completely destroyed. A few bacteria will remain viable somewhere or other in the organism, mainly in the lymph glands. Should the resistance of such animals be reduced for some reason or other the bacteria can begin to multiply strongly and evoke a fatal infection.

It has been shown that also in another member of the vole family, the vole rat, minimal doses of *M. tuberculosis* injected intravenously can provoke a tuberculous disease which ends in death at a late stage (*Jespersen* 1974). Two animals injected with eight viable units died on day 579 and day 1046 from pulmonary tuberculosis. Tubercle bacilli were found in the lymph glands by microscopy in the one case and by culture in the other.

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INFECTION OF *CLETHRIONOMYS G. GLAREOLUS SCHREB* (RED MICE) WITH *MYCOBACTERIUM BOVIS* INJECTED INTRAVENOUSLY

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Jespersen, A., Bentzon, M. W. & Møller, S. Infection of *Clethrionomys g. glareolus Schreb* (red mice) with *Mycobacterium bovis* injected intravenously. Acta path. microbiol. scand. Sect. B, 85: 415–426, 1977.

Groups of red mice were injected intravenously with doses of from 3 viable units to 3×10^7 viable units of a virulent strain of *M. bovis* grown in Dubos Tween medium and exposed to ultrasonics. The course of the infection was evaluated by the survival times of animals that had died spontaneously and by quantitative culture from the organs of animals injected with the four smallest doses and killed at various times after the injection. *M. bovis* provoked progressive, fatal infection in all animals, even in those injected with 3 viable units. The median survival times of the animals injected with the three largest doses were 15, 17 and 18 days, increasing the lower the dose. For the animals injected with the three smallest doses, the median survival times were all about 3 months, independent of dosage. On autopsy tuberculous lesions were found in spleen, lungs and lymph glands, but never in the liver. These lesions, which rapidly became caseous, were largest in the animals injected with a small dose, particularly in those which survived the longest. Smears from the organs contained enormous numbers of tubercle bacilli. Quantitative culture from the organs showed that the tubercle bacilli multiplied strongly during the first 3–4 weeks after the injection. Multiplication then ceased or became strongly inhibited. During the following 3 weeks, the number of viable units in the various organs remained at about the same level, being greatest in the spleen, smaller in the liver and lymph glands, and smallest in the lungs. During the rest of the experimental period, the number varied considerably from animal to animal, but generally it was almost unchanged in the lymph glands, liver and spleen, but increasing in the lungs. In some of the animals that lived longer than 3 months, the number of viable units in all the organs was extremely great.

Key words: *Mycobacterium bovis*; intravenous infection; *Clethrionomys g. glareolus Schreb* (red mice).

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Received 17.viii.77 Accepted 17.viii.77

A recently published work (*Jespersen et al.* 1977) showed that red mice were very resistant to *M. tuberculosis* injected intravenously. With a frequency varying according to the size of the dose, the bacilli induced pro-

gressive infection. However the course was extremely slow and in animals injected with doses of 10^4 viable units and less, the infection had no significant influence on the survival of the animals. In the present experiment, the infection of red mice with *M. bovis* has been evaluated after injection of varying doses, both by the survival times and by quantitative culture from the organs at various times after the injection.

MATERIAL AND METHODS

The experiment was carried out according to the model described previously for the infection of red mice with *M. tuberculosis* (*Jespersen et al.* 1977).

Experimental

a) *Survival times after injection of varying doses.* Groups of 20 red mice were injected intravenously with dilutions of 10^0 to 10^{-7} of a virulent strain of *M. bovis* grown in Dubos fluid Tween medium and dispersed by means of ultrasonics. The experiment also included a group of 20 animals which were not injected. All the animals were allowed to live until they died spontaneously, the experiment being concluded eleven months after injection. On autopsy smears were made from lymph glands, liver, spleen and lungs.

b) *Quantitative culture from organs at different times after injection of various doses.* Groups of red mice were injected intravenously with dilutions 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} of the same bacterial suspensions as those used in experiment a. 24 hours after the injection and then every fourth to fifth day during a period of up to 97 days (54 days for the 10^{-4} group) one animal from each group was killed. On autopsy the lumbar and mesenterial glands, the left liver lobe, the whole spleen and the left lung were taken out under sterile conditions, and the number of viable units was determined by quantitative culture. The lymph glands were pooled.

Experimental animals. The red mice, which were bred at the farm belonging to Statens Seruminstitut, were 2–5 months old when used in the experiment. They were distributed at random into earthenware jars, with one mouse in each. Each experimental group consisted of equal numbers of females and males.

Infection. A strain of *M. bovis* V 5157 B, which had recently been passed through a rabbit, was cultured on Löwenstein-Jensen medium and then twice in Dubos fluid Tween medium. Exposure of the culture to ultrasonics and preparation of dilutions for injection of the animals have been described previously (*Jespersen et al.* 1977). Inoculation from three suitable dilutions on to ten Löwenstein-Jensen tubes showed that 0.2 ml of 10^{-7} i.e. the smallest dose per mouse contained 3 viable units. The mice were injected into a tail vein with 0.2 ml bacterial suspension, starting with the smallest dose, then the ten times larger dose and so on. The mice to be given the individual doses were injected in random order. The inoculation process was carried out in just under 3 hours.

Quantitative culture. The organs were comminuted by grinding in mortar with the dropwise addition of 2 ml diluted Sauton. From that suspension (10^0) dilutions of 10^{-1}, 10^{-2} etc. were prepared with diluted Sauton medium, and 0.1 ml from three suitable dilutions was inoculated on to each of four Löwenstein-Jensen tubes.

Registration. The degree of tuberculosis and the number of bacteria in the organs of the animals in experiment a were recorded by means of an index based on macroscopical and microscopical findings and the results of culture.

Tuberculosis Index

0 = organs normal, culture negative
1 = no definite specific lesions, culture positive
2 = small tuberculous lesions, mainly regressive
3 = slight progressive tuberculosis
4 = medium progressive tuberculosis
5 = severe progressive tuberculosis

Bacterial Index

0 = no bacteria
1 = microscopy negative, culture positive
2 = 1–20 bacteria by microscopy (fluorescence microscopy)
3 = 21–99 bacteria
4 = 5–30 bacteria per sight field
5 = > 30 bacteria per sight field

Virulence determination of strain V 5157 B. Two groups of two guinea pigs were injected intraperitoneally with 1 ml of dilutions 10^{-} and 10^{-6} and

TABLE 1. *Virulence Determination of a Strain of M. bovis on Guinea Pigs and Rabbits*

	Viable units	Survival time (days)	Degree of tuberculosis
Guinea pig	14×10 i.p.	107	5*)
	14×10 –	129	5
	14×10^3	57	5
	14×10 –	75	5
Rabbit	14×10^4 i.	33	5
	14×10^5	18	5

*) 5 = severe generalized tuberculosis.

two rabbits were injected intravenously with 1 ml 10^{0} and 10^{-1} of the same suspensions as those used for injection of the mice. The results (Table 1) show that the strain was highly virulent for rabbits and guinea pigs.

Statistical Methods

Pairwise comparisons between the distributions of the survival times for doses 10^{-7} to 10^{0} were carried out by Wilcoxon's (1945) two-sample test. P-values < 5 per cent were considered significant.

For estimation of the number of bacteria in the various organs, a special method was used. This is described in a statistical appendix.

Only one animal was taken out each time for culture from the organs, and thus a direct estimate of the random variation from animal to animal cannot be made. Since corresponding animals injected with the three lowest doses were examined on the same days, the random variation can be estimated by the residual variance in a two-way analysis of variance, provided the curves run parallel. Such analyses have been made for two periods, i.e. days 26–54 and days 57–97 for each organ separately in order to see whether there was significant difference between the doses and between the times within the period.

RESULTS

Survival Times

Table 2 shows the survival times in days of red mice injected with doses of from 3 to 3×10^{7} viable units, and the survival times of a non-injected control group. The tuberculosis index and bacterial index for the individual animals in the injected groups are also given. Fig 1 shows the survival times in graphical form, with the percentage number of animals still alive at the various times plotted on the ordinate and days after injection on the abscissa.

Two of the animals in the non-injected control group died on day 106 and day 307 and the remainder were killed on day 327. None of the controls showed signs of tuberculosis, despite the fact that they had been placed at random among the infected animals.

In all the animals injected with *M. bovis* even those given only 3 viable units, a progressive fatal infection developed. The median survival times of the animals injected with the three highest doses were 13, 17 and 18 days, increasing the lower the dose. The animals in the 10^{-} group had a median survival time which was significantly longer than that in the 10^{0} group ($p < 0.1$ per cent) and the same was the case for the animals in the 10^{-2} group in relation to the 10^{-} group ($p = 0.2$ per cent). For the animals injected with the three smallest doses, the median survival times were about 3 months, irrespective of dosage.

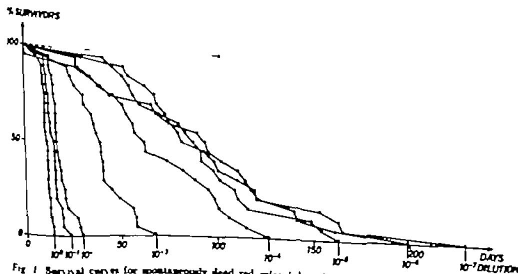


Fig. 1. Survival curves for spontaneously dead red mice injected intravenously with doses from 10^{0} to 10 (3×10^{7} to 3 viable units) *M. bovis* and group of non-infected control animals.

TABLE 2 *Survival Times Tuberculosis and Bacterial Indices for Spontaneously Dead Red Mice Inje – Times for a Non Infected Control Group Figures Given at the Bottom* _

Non-infected controls	10^{-7} 3			10^{-6} 3×10^{1}			10^{-5} 3×10^{2}			10^{-4} 3×10^{3}		
	Surv time (days)	Degree of tub	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index
106	33	3	2.3	30	3	2.8	44	4	4 0	1	0	0
309	55	4	2.5	30	3	2 0	49	5	4.5	31	4	3.5
k 327	57	3	2.5	36	4	3 8	54	5	4 8	37	5	5.0
k 327	65	5	4 8	42	5	4 8	57	5	4.8	41	4	3 0
k 327	70	5	5 0	47	5	4.3	59	4	4.3	49	5	4 8
k 327	71	5	5 0	68	5	5 0	62	4	4.5	53	4	3.8
k 327	75	5	3 8	71	5	4.3	72	4	3 0	55	4	3 0
k 327	77	4	3.3	77	5	4 8	82	5	3 0	58	5	3.5
k 327	81	5	4.0	92	5	4 0	85	5	4.3	59	5	5.0
k 327	83	4	3 8	95	5	4 0	89	5	4.0	64	5	4.3
k 327	94	5	4.8	96	5	3 8	93	4	2.5	64	5	4.8
k 327	99	5	3 3	103	5	4.3	93	5	2.8	75	5	4 0
k 327	101	5	4.5	111	5	1 8	99	5	4 8	83	5	3.5
k 327	111	5	4 0	115	4	2.3	102	5	3.5	89	5	2.3
k 327	120	5	2.8	118	5	4.5	110	5	3.0	96	5	4.3
k 327	121	4	?.8	122	5	5 0	111	5	4.5	99	5	3.8
k 327	154	5	4 0	140	5	3 0	116	5	3 0	101	5	3.8
k 327	163	5	4.5	141	4	3 0	149	5	4.3	106	5	2.8
k 327	165	5	3.5	161	5	3.3	154	5	3 8	115	5	3.5
k 327	229	5	4.5	199	5	4.0	163	5	4 0	127	5	4.3
>327	89		3 8	96		3 7	91		3 9	64		3 7

The infection had an intermediate course in the 10^{-5} and 10^{-4} groups, the median survival times being 41 and 64 days. These are mutually significantly different (p = 0 1 per cent) and also different from the groups injected with the three largest doses. The animals in the 10^{-4} group had a shorter median survival time than those in the 10^{-5} group but the difference was not significant (p = 9 per cent)

Autopsy findings The tuberculous lesions were dependent on dosage and were most prominent in the animals that survived the longest after being injected with the smallest doses. The frequency of enlarged lymph glands and of tubercles in spleen and lungs in the various groups is shown in Table 3 In the groups injected with the three largest doses, the lymph glands were slightly enlarged in almost all the animals, but only in exceptional cases were they caseous. Tubercles in the generally enlarged spleen were found in only four out of 60 animals. The liver was normal. The lungs contained tubercles in about half of the animals, the frequency being somewhat lower in the 10^{0} group where the survival times were the shortest. In the groups injected with the three smallest doses, practically all the lymph glands were enlarged up to the size of peas, and a quarter to half of them were caseous. In the strongly enlarged spleen there were tubercles in 75 to 90 per cent of the animals and it was an exception that they were not caseous. In cases where the course was protracted, larger or smaller irregularly formed sections of the spleen could be completely caseous, or hazel nut sized, distended, thin walled abscesses could be seen.

In the liver tubercles were found in the second month, but not later in the animals

[...]travenously with Varying Doses (Dilution Viable Units) of a Strain of M. bovis, Together with the Survival [...] Table are the Median Survival Times and the Average Bacterial Indic[es]

10^{-4} 3×10^{4}			10^{-3} 3×10^{5}			10 3×10^{6}			10^{-1} 3×10^{7}		
Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index
7	3	2.3	15	4	4.5	12	4	3.8	8	4	4.3
25	4	3.8	15	5	5.0	12	4	3.8	11	4	4.3
26	4	4.0	16	4	4.0	13	5	4.5	11	5	4.[illegible]
28	4	4.3	17	4	3.8	13	4	4.5	11	5	4.5
33	5	4.8	17	4	3.8	14	5	4.8	12	5	5.0
33	5	5.0	18	4	4.3	14	4	4.3	12	5	4.0
37	4	3.0	18	4	4.3	14	4	4.3	12	5	5.0
38	5	5.0	18	5	4.8	15	5	4.3	12	5	5.0
39	5	4.8	18	5	4.8	15	5	5.0	12	5	5.0
41	5	4.8	18	5	4.3	17	5	4.5	13	4	4.5
41	4	4.8	18	5	4.8	17	5	5.0	13	4	4.5
42	5	5.0	19	4	4.8	17	5	4.8	13	5	4.8
42	5	4.8	20	5	4.8	17	5	5.0	13	4	4.5
42	5	4.8	20	5	4.8	17	4	4.5	13	5	5.0
[illegible]	4	4.8	22	5	4.3	17	5	4.8	13	5	4.8
50	5	4.8	22	5	4.8	17	5	4.5	14	5	4.8
57	4	4.0	23	5	5.0	20	5	5.0	14	5	4.8
58	5	4.5	28	5	4.8	20	5	5.0	14	5	5.0
58	5	3.8	29	5	5.0	20	5	4.0	15	5	5.0
63	5	5.0	30	5	5.0	24	5	5.0	15	5	5.0
41		4.4	18		4.6	17		4.6	13		4.7

[...]ed for quantitative culture but this was not the case in those used for determination of the survival times. In almost all the animals there were tubercles in the lungs, and more than half of them were caseous. Sometimes a lung lobe or a whole lung could be transformed into a whitish-yellow caseous mass.

The groups injected with moderate doses, 10⁻ and 10⁻⁴ resembled generally the groups given the three smallest doses

Number of bacteria in organs The number of bacteria in the organs of the individual animals was evaluated by microscopy of smears and recorded as a bacterial index. The means of the indices for lymph glands, liver spleen and lungs in the various injection groups are shown in Table 4

In the groups injected with the three largest doses, the indices are maximal for the spleen and almost maximal for the liver The lungs and lymph glands contained somewhat fewer bacteria.

In the groups injected with the three smallest doses, the index for the liver is considerably lower for lymph glands and spleen slightly lower and for the lungs at the same level as in the groups injected with the three largest doses. The indices for the 10⁻⁴ and 10 groups resemble the corresponding values for the groups injected with the three largest and the three smallest doses, respectively

Quantitative Culture from the Organs

Infection dose 10^{-4} 3×10^{4} viable units (Fig. 2) In this and the subsequent figure, the logarithm of the number of viable units in lymph glands, liver spleen and lungs is plotted on the ordinate and days after injection on the abscissa. Tubercle bacilli could be found in liver and spleen after 24 hours, in

TABLE 5 *Frequency of Enlarged Lymph Glands and Tubercles in Spleen and Lungs in Groups of Red Mice Injected Intravenously with Varying Doses (Dilution Viable Units) of a Strain of M bovis*

	Lymph glands			Spleen			Lungs		
	Not enlarged	Enlarged not caseous	Enlarged caseous	No tubercles	Tubercles, not caseous	Caseous tubercles	No tubercles	Tubercles, not caseous	Caseous tubercles
10^{0} 3×10^{7}	1	18	1	19	0	1	13	7	0
10 3×10^{6}	2	18	0	19	0	1	8	10	2
10^{-2} 3×10^{5}	1	18	1	18	0	2	9	9	2
10^{-} 3×10^{4}	0	11	9	8	1	11	3	10	7
10^{-} 3×10^{3}	1	11	8	5	2	13	2	10	8
10^{-5} 3×10^{2}	1	14	5	4	2	14	0	11	9
10^{-6} 3×10^{1}	0	14	6	5	3	12	1	6	13
10^{-7} 3	0	10	10	2	0	18	3	5	12

TABLE 4 *Mean Bacterial Indices for Lymph Glands, Liver, Spleen and Lungs in Groups of Red Mice Injected Intravenously with Varying Doses (Dilution, Viable Units) of Strain of M. bovis*

Dose	Lymph glands	Liver	Spleen	Lungs	Mean of the four organs
[illegible] 3×10^7	4.4	4.9	5.0	4.5	4.7
[illegible] 3×10^6	4.1	4.9	5.0	4.2	4.6
[illegible] 3×10^5	4.4	4.8	5.0	4.1	4.6
[illegible] 3×10^4	4.6	4.3	4.8	3.9	4.4
[illegible] 3×10^3	3.9	2.5	4.0	4.1	3.6
[illegible] 3×10^2	4.1	2.8	4.3	4.5	3.9
[illegible] 3×10^1	3.8	2.6	4.1	4.4	3.7
[illegible] 3	4.5	2.9	4.0	4.0	3.8

the lungs on day 5 and in the lymph glands on day 13. The bacteria multiplied strongly in all organs during the first 3 weeks. The number which reached a higher level than in the animals injected with the same dose of *M. tuberculosis* was larger in liver and spleen than in lymph glands and lungs. From day 26 and during the rest of the experimental period —which was only 54 days for this dose—the number of bacteria remained almost unchanged in liver, spleen and lymph glands, while it had a tendency to increase in the lungs. The curves for lymph glands and lungs lay constantly below the curves for liver and spleen.

Infection doses 10^{-5}, 10^{-6} *and* 10^{-7} (3×10^2, 3×10^1 *and 3 viable units*) (Fig. 3). The experimental period was 97 days. The results of culture were quite uniform in the animals injected with these three doses and thus can be described together.

In the 10 group, tubercle bacilli were found for the first time in the liver on day 1, in the spleen on day 5, in the lungs on day 9 and in the lymph glands on day 13. The corresponding times in the 10 group were days 5, 13, 13 and 21. The bacteria multiplied strongly in the organs of all animals until day 26. From day 26 to day 54 the number of viable units for the individual organs was at about the same level, the curve for the spleen being highest, followed by the curves for liver and lymph glands, and lowest the curve for the lungs. During the rest of the experimental period, the number varied considerably from animal to animal, and in the individual animals there were often more bacteria in the lungs than in the other organs. Generally, the number of viable units in the lymph glands, liver and spleen was almost unchanged from day 26 to the end of the experimental period, while it was increasing in the lungs. In animals where the survival times were protracted, the organs contained very large numbers of bacteria.

Statistical Analysis

The course of the curves for the three lowest doses was uniform, and therefore a mean curve was calculated for each organ for these doses. Two-way analysis of variance for each organ in the periods days 26–54 (period 2) and days 57–97 (period 3) gave the following. The variation between the animals was greater in period 3 than in period 2. The ratio between the variance estimates was 2.0, 4.4, 5.8 and 2.3 for lymph glands, liver, spleen and lungs, respectively. For liver and spleen, the values are significantly greater than 1 ($p < 0.1$ per cent in both cases).

On the whole, no difference could be found between the days within the periods; only for period 3 was there a significant difference between the days as regards the lungs. Differences in dose could be found in period 2 for liver and spleen. The mean level was the same in the two periods for lymph glands,

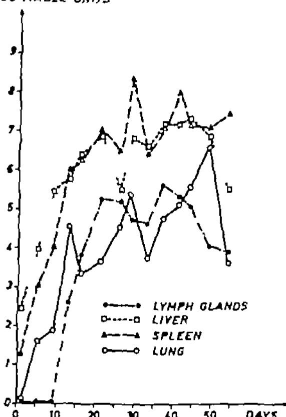


Fig 2 Dose 10^{-4} 3×10^{3} viable units.

liver and spleen while for the lungs there was a significantly higher level in period 3

During the latter part of period 3 the culture results were affected by the fact that some of the animals, which according to schedule should have been taken out for autopsy were already dead. Thus a selection occurred which must be considered to have affected the results towards too small numbers of bacteria.

The calculations show that for lymph glands and liver there was a tendency towards smaller numbers in the period after 82 days, while for the lungs there was a tendency towards higher figures. However the differences were not significant in any of the cases.

In the figures, the points for days 75 and 82 are joined together with a thinner stippled line than the others.

DISCUSSION

Survival times *M bovis* injected intravenously into red mice provokes a progressive infection which rapidly results in death. Even the smallest dose 3 viable units, caused an in-

Figs 2–3 Number of viable units in organs from red mice injected with doses from 10^{-4} to 10^{-7} *M bovis* and killed at various times after injection. Ordinate log viable units. Abscissa days after injection.

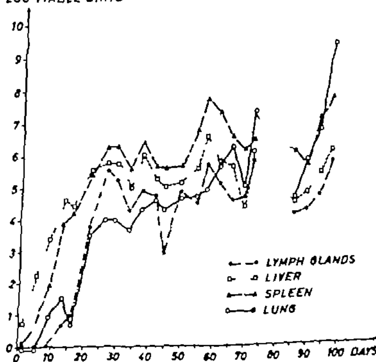


Fig 3 Average curves for doses 10^{-5} 10^{-6} and 10^{-7} *M bovis* 3×10^{2} 3×10 and 3 viable units.

fection to all the animals in the group. A previous experiment (*Mackeprang & Jespersen* 1959) with red mice injected with ultrasonic-exposed and non-exposed Dubos culture of *M. bovis*, has shown that a single viable unit is sufficient to provoke fatal tuberculosis.

During the infection, severe, often caseous, lesions develop in the organs, particularly in the lymph glands, lungs and spleen. The number of bacteria in the organs is very large, particularly in the caseous lesions where it is enormous. The vole rat is the only one of the other voles which has been infected with tubercle bacilli by the intravenous route (*Jespersen* 1974). Doses of from 7 to 730 viable units of a strain of *M. bovis* provoked fatal infection with survival times from 148 to 268 days. The autopsy findings resembled those in red mice, except that generally the liver contained caseous tubercles and the lymph glands were even more enlarged.

Quantitative culture. An evaluation of the method has been carried out previously (*Jespersen et al.* 1977). The bacterial suspension injected consisted of 75 per cent units with one or two bacteria, and none of the remaining units contained more than 10 bacteria. On account of the stronger multiplication of *M. bovis* more and larger bacterial clumps develop in the organs than in mice injected with *M. tuberculosis*. Since the clumps cannot be separated during comminution in mortar and since both a single bacterium and a clump will grow into one colony, the number of colonies will be smaller than the number of bacteria. The difference will be greatest in the *M. bovis* infection (*Jespersen*, unpublished).

As in the *M. tuberculosis* infection (*Jespersen et al.* 1977) the course of the infection can be divided into three phases, but in this case the second phase was shorter and consequently the difference between the second and third phases was less marked.

The bacteria multiplied strongly in the organs during the first 3 to 4 weeks after the injection (first phase), after which, as a result of the immunity developed, multiplication stopped or became strongly inhibited. At the end of the first phase the number of viable units was considerably larger than in the corresponding phase of the *M. tuberculosis* infection. The initial phase of multiplication was followed by a period of about 4 weeks (second phase) during which the number of viable units in the various organs was almost constant in the individual animals. The curve was highest for the spleen, lowest for the lungs and intermediate for liver and lymph glands. Neither did the autopsy findings show any particular difference in the individual animals during that period, a phenomenon which has also been observed in other animal species (*Ratcliffe & Palladino* 1953). In the third phase, from day 57 to the end of the experimental period, there was considerably greater variation from animal to animal in both culture results and autopsy findings than in the second phase, thus reflecting variation in the resistance acquired by the individual mice. Taking the second and third phases together it has been shown that the number of viable units in lymph glands, liver and spleen was almost unchanged, while it was increasing for the lungs. This does not mean that the bacteria cannot multiply in the third phase. Among the animals that lived longer than 3 months, there were often cases where the number of viable units in the organs was very great, especially in lymph glands and lungs.

As mentioned above, the multiplication of bacteria in the first phase was stronger than in the corresponding phase of the *M. tuberculosis* infection. The acquired resistance induced is consequently stronger and presumably as high as it can be in red mice. Nevertheless it was not able to prevent the development of a fatal tuberculous disease, not even in animals injected with the smallest dose (3 viable units). The only effect of which the immunity was capable was to stop or inhibit multiplication of bacteria for a time and to destroy some of the bacteria in the organism, particularly in the liver.

Lurie (1928), who examined the course of infection in rabbits after intravenous injection of *M. bovis* by quantitative culture from the organs, found that the bacteria multiplied in the organs until the fourth week. During the

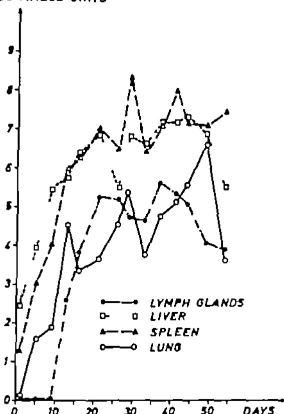


Fig 2 Dose 10^{-4} 3×10^3 viable units.

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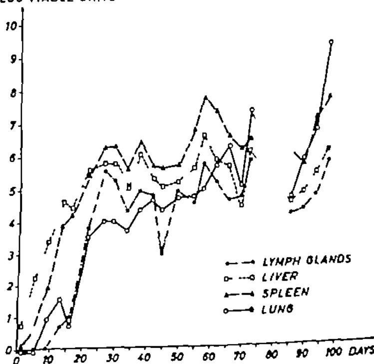


Fig 3 Average curves for doses 10^{-5} 10^{-6} and 10^{-7} *M bovis* 3×10^2 3×10^1 and 3 viable units.

$\frac{1}{10} K_{10}$

LYMPH GLANDS
□ LIVER
▲ SPLEEN
○ LUNG

K_1

Fig 1 a. M bovis. Dose 3×10^{3} viable units.

$\frac{1}{10} K_{10}$

LYMPH GLANDS
□ LIVER
▲ SPLEEN
○ LUNG

K_1

Fig 1 b M tuberculosis Dose 11×10^{3} viable units.

Appendix Fig 1 a and 1 b Comparison between colony counts K_{10} and K_1 for two successive dilutions. The lines correspond to = 0 0.001 0.002 and 0.003 respectively

second month the number of tubercle bacilli in the spleen and particularly in the liver decreased while multiplication continued in lungs and kidneys, and the animals soon died of pulmonary and renal tuberculosis. Thus there is accordance on important points between *Lurie's* and the writer's experiments.

Ratcliffe (1952) and *Ratcliffe & Palladino* (1953) found that the first phase of the infection with *M. tuberculosis* or *M. bovis* develops similarly in various animal species. In rats, mice, hamsters and guinea pigs, which inhaled single bacteria in fine drops the *number of initial tubercles which developed corresponded almost to the number of bacteria* inhaled. The tubercles developed to macroscopical size at apparently the same speed in all animals during the course of 4 weeks, after which the speed and pattern varied greatly in the individual species. The authors concluded that the native resistance to tubercle bacilli in these animal species is not different, but that they have a widely varying capacity to develop acquired resistance.

In the present and the previous works, a difference has been found in the native resistance to the two bacterial species. At the end of the initial phase of the *M. tuberculosis* infection the number of viable units in the organs from animals injected with the two smallest doses was considerably less than in animals injected with corresponding doses of *M. bovis*.

STATISTICAL APPENDIX

The number of viable bacterial units in the organs is determined on the proviso that the various suspensions 10^{-1}, 10^{-2} etc. contain 1/10, 1/100 etc. of the number of viable units in the suspension 10^{0}. For each dilution, the number of colonies is counted on four tubes and the total number of colonies per dilution is used in the calculations. Out of the number of colonies observed in the suspensions, generally only two will fall within the range 5–200. If, for instance, the colony counts for 10^{-5} and 10^{-6} are kalled K_{10} and K_1, it would be expected that, with the exception of random variations

(1) $K_1 = 1/10\, K_{10}$.

This expectation can be tested by plotting such a pair of colony counts on a diagram with $1/10\, K_{10}$ on the ordinate and K_1 on the abscissa. Figs. 1 a and 1 b show the pair observed for 10^{-4} *M. bovis* and 10^{-4} *M. tuberculosis*. It will be seen that there is systematic deviation from (1). A similar result has been described previously by *Engbæk et al.* (1967) where it was stated that the expected number of colonies (x) was

$$(2)\quad x = \frac{\lambda \times F}{1 + \alpha\lambda F}$$

where λ is the number of bacterial units per ml for suspension 10^{0}, F the dilution factor and $\alpha \geq 0$ the parameter which determines how much x is less than λ.

For the example in question

$$(3a)\quad K_{10} \approx x_1 = \frac{\lambda \times 10^{-5}}{1 + \alpha\lambda \times 10^{-5}}$$

and

$$(3b)\quad K_1 \approx x_1 = \frac{\lambda \times 10^{-6}}{1 + \alpha\lambda \times 10^{-6}}$$

From (3b) it obtains that

$$(4)\quad \lambda \approx \frac{10^{6} K_1}{1 - \alpha K_1}$$

which, inserted into (3a) gives

$$(5)\quad \frac{1}{10} K_{10} \approx \frac{K_1}{1 + 9\alpha K_1}$$

It will be seen from the curves that α will lie between 0.001 and 0.003.

Using (3a) and (3b) it is possible to calculate values for λ and α for a given pair of colony counts. It does not seem justifiable to determine a new value for α for each new pair of counts, since according to the model, α is not dependent on λ. It was therefore sought to find a value for α which is common for a number of counts. As model for the distribution of colony counts, the negative binomial distribution is used

$$(6)\quad p(K) = \binom{\gamma + K - 1}{K} \left(\frac{x}{\gamma + x}\right)^{K} \left(\frac{\gamma}{\gamma + x}\right)^{\gamma}$$

For this it applies that

Acta path. microbiol. scand. Sect. B 85 427-434 1977

ON THE DIAGNOSIS OF COAGULASE-NEGATIVE STAPHYLOCOCCI WITH EMPHASIS ON *STAPHYLOCOCCUS SAPROPHYTICUS*

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Hovelius, B. & Mårdh, P.-A. On the diagnosis of coagulase-negative staphylococci with emphasis on *Staphylococcus saprophyticus*. Acta path. microbiol. scand. Sect. B 85: 427-434, 1977.

This study concerns the diagnosis of coagulase-negative staphylococci, with special emphasis on novobiocin-resistant species, i.e. *S. saprophyticus*, *S. cohnii* and *S. xylosus*. Disc diffusion tests for novobiocin were found useful in the differential diagnosis of coagulase-negative staphylococci isolated from urine specimens, but not from pus and blood cultures. We report on the resistance of *S. saprophyticus* to nalidixic acid and the use of this characteristic in the diagnosis of coagulase-negative staphylococci known to be novobiocin-sensitive, but which have subsequently acquired resistance to novobiocin. The results of different tests for betalactamase production in *S. saprophyticus* are presented. "Clover leaf" tests suggested such a production in about half of the strains studied, while no strain produced betalactamase as indicated by tests using chromogenic cephalosporin or benzylpenicillin in capillary tube tests.–The failure of tests for nitrate reduction, glucose consumption and of cultures of urine on MacConkey's agar in the diagnosis of urinary tract infections caused by *S. saprophyticus*, is documented. The concept "significant bacteriuria" in the diagnosis of *S. saprophyticus* infections of the urinary tract above the bladder neck is also considered.

Key words: Coagulase-negative staphylococci; *Staphylococcus saprophyticus*; diagnosis; nalidixic acid; urinary tract infections.

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Received 10.vi.77 Accepted 5.ix.77

According to previous practice, coagulase-negative, novobiocin-resistant staphylococci were classified as micrococci, belonging to *Micrococcus* subgroups 1-IV (2). However, the guanine-cytosin (GC) contents (20) and the chemical composition of the cell wall (17) of these bacteria indicate that the organisms ought rightly to be included in the genus *Staphylococcus*. According to Bergey's manual (3), coagulase-negative, novobiocin-resistant staphylococci isolated from urine specimens should be classified as *Staphylococcus saprophyticus* biotype 3. The *Subcommittee* (22) proposed that the coagulase-negative, novobiocin-resistant staphylococci be separated into three species, i.e. *S. cohnii*, *S. saprophyticus* and *S. xylosus*, a recommendation based on studies by *Kloos & Schleifer* (10). Recently these latter authors have

(7a) $M(K) = x$

and

(7b) $V(K) = x + \frac{1}{\gamma} x^2$

On the basis of the counts on the four tubes for each dilution, the mean number of colonies $\bar{K}$ and $s^2_K = \frac{1}{3} \Sigma (K-\bar{K})^2$ is calculated According to (7a) and (7b) it should then apply that

(8) $$\frac{1}{\bar{K}} \times s^2_K \approx 1 + \frac{1}{\gamma} K$$

Using this relation it is found that $\frac{1}{\gamma}$ is about 0 060 For a sum S of n colony counts it applies that

(9) $$V(S) = \sigma + \frac{1}{\gamma} \sigma^2$$

where $\sigma = n\,x$ and $\gamma_s = n\,\gamma$ The calculations use $\gamma_4 = 4 \frac{1}{0\,060} = 66\,7$ With this value for γ an estimate can be made by the maximum likelihood method of a value for α which is common for a number of pairs of colony counts and a value for λ for each pair

It has not been possible to show any systematic difference in the α value between counts of viable units of *M bovis* or *M tuberculosis* or between culture counts from various organs. The value for α which gave the best fit was $\alpha = 0\,002$. Using this value the maximum number of colonies will be $1/\alpha = 500$ In Figs. 1a and 1b the middle curve corresponds to formula (5) with $\alpha = 0\,002$ Despite the large variations, it will be seen that the curve gives a better fit than the straight line corresponding to $\frac{1}{10} K_1 = K_1$

In the choice of the two colony counts K and K_1 which form the basis of the calculations it has been taken into account that it is important to avoid colony counts below 4 and over 200 (1 and 50 per tube) If K_1 is ≥ 400 λ is calculated from K_1 alone by means of formula (4) with $\alpha = 0\,002$ The relationship between λ and K_1 is illustrated by the following table

K_1	λ	λ/K_1
0	0	–
10	10.2	1 02
20	20 8	1 04
50	55 6	1 11
100	125 0	1.25
200	333.3	1 67
400	2000 0	5 00
500	∞	∞

In the few cases where K_1 is also ≥ 400, λ is set at the value corresponding to $K_1 = 400$ If both K_1 and K_{10} are < 400 λ is determined as described above.

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n nalidixic acid by disc diffusion tests. The tests were made as described above, though using paper discs containing 30 µg of nalidixic acid (AB Biodisk). In addition, all the 25 novobiocin-resistant strains of *S. epidermidis* and the eight strains of *S. cohnii* and *S. xylosus* isolated from the urine specimens, pus and blood cultures were tested for their susceptibility to nalidixic acid by the same technique.

Tests for Betalactamase Production

Fifty of the strains of *S. saprophyticus* which had been isolated from urine specimens were tested for betalactamase production by the aid of "clover leaf" tests in which one strain of *Diplococcus pneumoniae* and one strain of *Staphylococcus aureus* were used (15). Tests for production of such enzymes were also performed in capillary test tubes using benzyl-penicillin (16) and with the aid of chromogenic cephalosporin (Compound 87/312, Glaxo Research, Ltd. Greenford, England) (14). Of the latter compound, 0.5 mg was dissolved in 1 ml of 0.05 M phosphate buffer pH 7.0. The strain of *S. saprophyticus* under test was rubbed over the surface of filter paper which had been soaked with the cephalosporin solution. The same strains which had been grown in tryptone broth in the presence of benzyl-penicillin (0.2–0.16 µg per ml) were also tested by the three tests mentioned.

Chemical Screening Tests for Bacteriuria

Voided urine specimens containing *S. saprophyticus* at a density of $> 10^4$ bacteria per ml, as demonstrated in quantitative cultures of blood agar plates, were tested for nitrite (N-Labstix, Ames Co. England) and also for reduced glucose levels (Uriglox, Kabi, Stockholm). The tests were performed according to the instructions of the manufacturers.

TABLE 1. *Classification of Coagulase-negative Novobiocin-sensitive Staphylococci Isolated from Urine Specimens, Pus and Blood Cultures. Fifty Strains from each Type of Specimen were Tested*

Species	No. of strains of indicated species		
	Urine	Pus	Blood n
S. epidermidis	25	35	39
S. haemolyticus	3	4	1
S. hominis	3	4	7
S. simulans	12	6	2
S. warneri	5	1	1

RESULTS

Classification of Coagulase-negative Staphylococci Isolated from Urine Specimens, Pus and Blood Cultures

The classification of the coagulase-negative, novobiocin-sensitive strains isolated from urine, pus and blood cultures is shown in Table 1, while that of the novobiocin-

TABLE 2. *Classification of Coagulase-negative Novobiocin-resistant Staphylococci Isolated from Urine Specimens, Pus and Blood Cultures*

Species	No. of strains of indicated species		
	Urine (N = 150) n	Pus (N = 150) n	Blood (N = 150) n
S. epidermidis	1	10	14
S. cohnii		4	1
S. saprophyticus	49	2	
S. xylosus		5	

TABLE 3. *Biochemical Test of 50 Coagulase-negative Novobiocin-sensitive and 50 Novobiocin-resistant Staphylococcal Strains Isolated from Urine Specimens*

Test	Test positive for no. of strains	
	Novobiocin-sensitive*	Novobiocin-resistant**
Glucose)	50	1
Arabinose	0	0
Cellobiose	0	0
Galactose	33	5
Lactose	44	49
Maltose	42	50
Mannitol	18	49
Mannose	12	0
Sucrose	49	50
Trehalose	30	49
Turanose	26	49
Xylitol	0	48
Arginine dihydrolase	46	1
Phosphatase	11	5
Urease	38	50
Nitrate reduction	44	2

* MIC < 1.0 µg per ml.
** MIC > 1.0 µg per ml.
) Tests were performed under anaerobic conditions.

suggested that *S. sciuri* be included as a new species among the coagulase-negative novobiocin-resistant staphylococci (11).

S. saprophyticus is a common cause of acute cysto-urethritis in women, particularly during the early reproductive years (12). Although urinary tract infections (UTI) caused by this bacterium occur much less often in males, they are sometimes to be found in elderly men, and in boys and young adult men presenting with acute haemorrhagic cystitis. Recently we have demonstrated that *S. saprophyticus* frequently causes symptoms suggestive of an upper urinary tract infection and that recurrences of UTI with this organism do occur quite commonly (8).

In the present study methods used in the differential diagnosis of coagulase-negative novobiocin-resistant staphylococci isolated from urine specimens, pus, and blood cultures, are evaluated. Possible reasons for overlooking the laboratory diagnosis of UTI by *S. saprophyticus* are given. The diagnostic relevance of the concept "significant bacteriuria" in UTI caused by coagulase-negative staphylococci is also discussed.

MATERIALS AND METHODS

Media

The blood agar plates used consisted of Blood agar base No. 2 (Oxoid) with 4 per cent defibrinated horse blood. A dip-slide culture set, Uricult (Orion Diagnostica, Finland) with MacConkey's and CLED agar pads, was used as recommended by the manufacturer.

Clinical Specimens and Culture Techniques

Mid-stream portions of voided urine and urine specimens collected by suprapubic aspiration were studied. In 15 patients, suprapubic aspiration was performed 2–3 days after growth of *S. saprophyticus* had been demonstrated in a voided urine specimen. The bladder puncture was performed before any antibiotic treatment had been given.

All specimens were obtained after at least 4 h of bladder incubation. Quantitative cultures were made on blood agar plates by the calibrated loop technique (6). The plates were read after overnight incubation at 37 C. Except for the specimens from the 15 patients subjected to suprapubic bladder aspiration, only urine specimens which showed a growth of $> 10^4$ staphylococci per ml were studied.

Coagulase-negative staphylococci isolated from randomly selected pus specimens and blood cultures sent to our clinical bacteriological laboratory were also studied.

Techniques for Classification of Staphylococci

Altogether 450 non-coagulase producing (21) staphylococcal strains isolated from urine, pus and blood cultures (150 strains from each type of specimen) were studied. Tests with glycerol- and erythromycin-containing agar plates (18) were made in order to confirm that the strains studied were staphylococci and not micrococci. All novobiocin-resistant strains, i. e. 84 strains, were tested for their ability to ferment the following carbohydrates under aerobic conditions: arabinose, cellobiose, fructose, galactose, lactose, maltose, mannitol, mannose, ribose, sucrose, trehalose, turanose, and xylitol. The organisms were also tested for phosphatase and urease activity and for their ability to reduce nitrate (19). Finally glucose fermentation tests, performed under anaerobic conditions, were made as recommended by the *Subcommittee* (21), while tests for arginine-dihydrolase activity were made ad modum *Digranes & Oeding* (4). In addition, 150 of the altogether 366 novobiocin-sensitive strains (50 strains isolated from each of the three types of clinical specimens studied) were subjected to the tests mentioned above.

Antibiotic Susceptibility Tests

All staphylococcal strains were assayed by disc diffusion tests (5) for their susceptibility to novobiocin using paper discs containing 5 μg novobiocin and Wellco susceptibility test agar (AB Biodisk, Stockholm). Strains which gave an inhibitory zone of < 15 mm in diameter are referred to in the following text as "resistant" to novobiocin, in conformity with the terminology in current use in this context.

The minimum inhibitory concentrations (MIC) of nalidixic acid and novobiocin for 50 strains of *S. saprophyticus* and altogether 50 strains of coagulase-negative novobiocin-sensitive staphylococci, i. e. strains of *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. simulans* and *S. warneri* were studied. These 100 strains had all been isolated from urine specimens. MIC was determined by using agar plates containing 2-fold increasing concentrations of nalidixic acid (Winthrop) and novobiocin (Sigma). The plates were inoculated by means of a replicating device and incubated at 37 C for 18 h before being read. That concentration of the antibiotic which inhibited growth (except for single colonies) was regarded as the MIC. The 100 strains were also tested for their susceptibility

blood cultures, 24 were classified as *S epidermidis* while the remaining 10 strains belonged to *S saprophyticus S cohnii* or *S xylosus* Thus, the results indicate that tests for susceptibility to novobiocin can, with a high degree of accuracy be used to identify *S. saprophyticus* isolated from urine specimens, but not among coagulase-negative staphylococcal strains isolated from pus and blood cultures.

The results of the MIC determinations are shown in Fig 1 The MIC of the 50 strains of *S saprophyticus* to novobiocin and nalidixic acid were 12.8–51.2 and 128–512 µg per ml, respectively The MIC for these antibiotics of the other 50 coagulase-negative staphylococcal strains studied was 0 1–6 4 and 16–128 µg/ml, respectively The MIC to nalidixic acid of the 23 tested strains of *S epidermidis* which were resistant to novobiocin, varied between 16 and 128 µg per ml. The MIC to this antibiotic for the 8 strains of *S cohnii* and *S xylosus* was ≥ 512 µg per ml.

The results of the disc diffusion tests for susceptibility to nalidixic acid performed on the coagulase-negative staphylococci isolated from the urine specimens are shown in Fig 2 No growth inhibition was found in tests on 49 of 50 strains of *S saprophyticus* The 50 strains belonging to the other coagulase-negative staphylococcal species viz *S epidermidis*, *S haemolyticus S hominis S simulans* and *S warneri* produced inhibitory zones, except for one strain of *S epidermidis*. Nalidixic acid caused inhibitory zones when testing all but one of the 25 novobiocin-resistant strains of *S epidermidis* isolated from urine pus, and blood cultures. *S cohnii* and *S xylosus* isolated from pus and blood cultures were not inhibited by nalidixic acid

Thus, the result indicates that disc diffusion tests for nalidixic acid can be used to differentiate coagulase-negative, novobiocin-resistant staphylococci from coagulase-negative staphylococci of originally novobiocin-sensitive species but which have since acquired resistance to novobiocin, i e usually strains of *S epidermidis* Such strains were isolated from urine, pus specimens and blood cultures of in-patients, but never from specimens obtained from out patients.

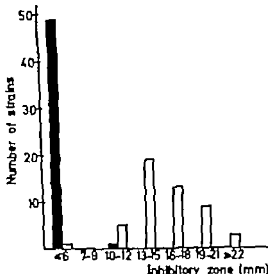


Fig 2 Diameters of inhibitory zones obtained in disc diffusion tests with nalidixic acid studying 50 strains of *Staphylococcus saprophyticus* (■) and altogether 50 strains of *Staphylococcus epidermidis*, *Staphylococcus haemolyticus Staphylococcus hominis Staphylococcus simulans* and *Staphylococcus warneri* (□) isolated from urine specimens.

Tests for Betalactamase Production by S. saprophyticus

None of the 50 strains of *S saprophyticus* produced betalactamases, as indicated by the test using chromogenic cephalosporin, and the capillary tube test using benzylpenicillin. However when the same strains were studied by "clover leaf" tests using *D pneumoniae* or *S aureus* the results suggested betalactamase production in 30 of the 50 strains tested. The same results were obtained when testing the same strains exposed to increasing concentrations of benzylpenicillin in liquid medium.

Chemical Tests for Bacteriuria and Dip-slide Cultures of Urine Specimens Containing > 10⁴ Coagulase negative Staphylococci per ml

Table 4 shows the results of chemical screening tests for bacteriuria, performed on

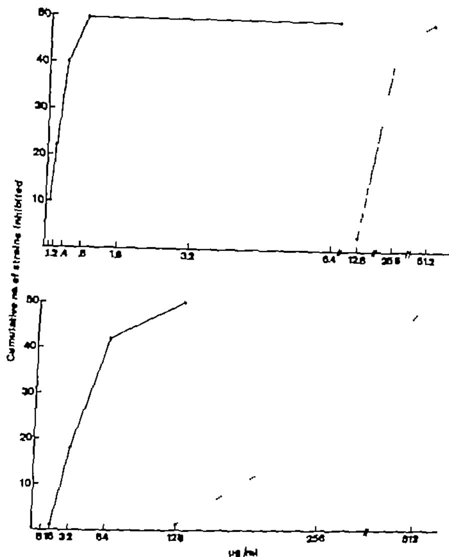


Fig 1 Susceptibility to novobiocin (upper panel) and to nalidixic acid (lower panel) of 50 strains of *Staphylococcus saprophyticus* (O —— O) and of altogether 50 strains of *Staphylococcus epidermidis, Staphylococcus haemolyticus Staphylococcus hominis Staphylococcus simulans* and *Staphylococcus warneri* (O——O) isolated from urine specimens. Cumulative numbers of strains inhibited by indicated concentrations (μg/ml)

resistant strains is shown in Table 2. The results of some of the biochemical tests made for the classification of the 100 strains of coagulase negative staphylococci isolated from urine specimens are presented in Table 3 All but one of the 50 novobiocin resistant strains tested showed roughly the same carbohydrate fermentation pattern and could be classified as *S saprophyticus*

Susceptibility Tests to Novobiocin and Nalidixic Acid in the Classification of Coagulase negative Staphylococci Isolated from Urine Specimens Pus and Blood Cultures

Of all the 150 coagulase negative strains isolated from the urine specimens, 33 per cent proved resistant to novobiocin while of the 150 staphylococcal strains isolated from the pus specimens and the blood cultures, 13 and 10 per cent respectively were resistant to this antibiotic Of the altogether 34 novobiocin resistant strains isolated from pus and

strain of *S saprophyticus* caused inactivation of benzylpenicillin in the "clover leaf" test by other means than betalactamases.

Among coagulase-negative staphylococci found on the skin of domestic animals and pets, novobiocin-resistant species of staphylococci predominate over novobiocin-sensitive ones (7). This also indicates that susceptibility tests for novobiocin cannot be used to differentiate coagulase-negative staphylococci in specimens obtained from animals, nor in specimens collected from persons having contact with animals in their profession, e.g. veterinarians and abattoir workers (13). We were able to frequently isolate coagulase-negative, novobiocin-resistant staphylococci from the hands of such categories of workers. It is notable that *S saprophyticus* could be isolated from only those slaughterers who had wounds on their hands (7). However further studies are required to establish whether *S saprophyticus* may play a pathogenic role in these wounds.

S saprophyticus may only occasionally be isolated from voided urine specimens of persons lacking symptoms and signs of UTI. The demonstration of even sparse growth ($< 10^4$ organisms per ml) of *S saprophyticus* in such specimens would appear to be a finding of clinical significance. This implies that staphylococci isolated from urine specimens even in numbers less than 10^4 per ml should be submitted to testing for coagulase production and if found coagulase negative, they should be tested for their susceptibility to novobiocin in order to identify *S saprophyticus*. This diagnostic procedure is only rarely undertaken, which is one of the reasons why UTI with *S saprophyticus* may escape diagnosis.

The demonstration of significant bacteriuria i.e. 10^5 bacteria per ml of the same species in two consecutive voided urine specimens (9) seems to be of limited value as a mean to differentiate infections by *S saprophyticus* above the bladder neck from contamination (1). The cultures of voided and bladder urine specimens showed only sparse or moderate growth of *S saprophyticus* i.e. $< 10^5$ bacteria per ml, in half of the patients studied in whom the organism had been recovered from suprapubically aspirated urine.

The determination of the number of bacteria in a urine specimen by quantitative cultures on agar plates is based on the assumption that the number of colonies obtained are directly related to the number of organisms present in the specimens studied. We have found that *S saprophyticus* has a pronounced tendency to agglutinate and to form aggregates in urine specimens (8). This implies that there might be a descrepancy between the true number of such organisms present in a urine specimen and that demonstrated by quantitative culturing.

The diagnosis of UTI by *S saprophyticus* is constantly missed when the diagnosis of UTI is based on tests for nitrite. *S saprophyticus* does not reduce nitrate. UTI with this organism may also escape diagnosis, if tests for reduced glucose levels in urine are employed as a screening test for UTI. As was also shown in our study infections with *S saprophyticus* may remain unrecognized if MacConkey's agar is used for culture of urine specimens, since the organism often fails to grow (or grows scantily and slowly) on this medium. It should be noted that MacConkey's agar is often used in dip-slides.

This study was supported by grant No 16X-4509 from the *Swedish Medical Research Council*

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voided urine specimens containing > 10^4 coagulase-negative staphylococci *per ml* as demonstrated by quantitative cultures on blood agar plates As expected all 50 urine specimens which had displayed growth of *S saprophyticus* reacted negatively when tested for nitrite by the N Labstix The Uriglox test did not indicate reduced glucose levels (< 2 µg per 100 ml) in 19 of the 40 specimens tested The table also sets out the results of parallel dip-slide cultures on MacConkey's and CLED agar (Uricult) The agar pads were read after 24 hours of incubation as recommended by the manufacturer *S saprophyticus* did not grow on the MacConkey's agar pad in 8 of the 30 urine specimens tested whereas the organism had been isolated on blood agar plates from all 30 specimens. However *S saprophyticus* could be isolated from all but one of these 30 specimens on the CLED agar pad

TABLE 4 *Urine Specimens Containing Staphylococcus saprophyticus (>10^4 Colony-forming Units per ml on Blood Agar) Tested for Nitrite Reduction (N Labstix) Glucose Consumption (Uriglox) and Growth on Dip-slides (Uricult)*

Test	No. of specimens tested	No. of tests indicating bacteriuria
N Labstix	50	0
Uriglox	40	21
Uricult		
MacConkey's agar	30	22
CLED agar	30	29

Quantitative Urine Cultures of Voided and Bladder Urine Specimens from Patients with UTI Caused by S. saprophyticus

In all 15 patients from whom *S saprophyticus* had been isolated from voided urine specimens and who had been subjected to bladder puncture, the bacterium could also be recovered from the suprapubically aspirated urine In 5 of the 15 bladder urine specimens, sparse (< 10^4 bacteria per ml) growth of *S saprophyticus* was found, while of the remaining 10 specimens two showed moderate and eight heavy growth (≥ 10^4 bacteria *per ml)*

DISCUSSION

As found in our study disc diffusion tests for novobiocin can be used with a high degree of accuracy to differentiate *S saprophyticus* from other coagulase-negative staphylococci in urine specimens However in the two other types of clinical specimens studied, viz. pus and blood cultures, staphylococci belonging to primary novobiocin-sensitive species, but which had acquired resistance to this antibiotic, were demonstrated frequently About 10 per cent of the strains of *S epidermidis* isolated from blood specimens were resistant to novobiocin. These novobiocin-resistant strains were often resistant to several other antibiotics too e.g betalactam antibiotics, sulphonamides and tetracyclines. It is notable that such strains were never recovered from specimens obtained from patients treated in primary health care.

All novobiocin resistant strains of *S epidermidis* had a MIC for nalidixic acid of 16–128 µg per ml. In this respect, these strains did not differ from novobiocin sensitive strains of *S epidermidis* thus indicating that susceptibility tests for nalidixic acid can be used to differentiate *S saprophyticus* and staphylococci of other primary novobiocin-resistant species, from coagulase negative staphylococci having acquired resistance to novobiocin

None of the strains of *S saprophyticus* tested were found to produce betalactamases, as indicated by the results of two of the three tests performed. However the "clover leaf" test, originally said to detect betalactamase production in staphylococci suggested such production in more than half of the strains tested However such enzyme production could not be demonstrated when using chromogenic cephalosporin and capillary tube tests with benzylpenicillin nor when testing the same strains exposed to benzylpenicillin An explanation of the described discrepancy is lacking but the finding suggests that some

Acta path. microbiol. scand. Sect. B, 85 435–439 1977

ERYTHROPOIETIC ACTIVITY AND INTERFERON PRODUCTION IN LCM VIRUS-INFECTED NUDE MICE

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Christoffersen, P J & Bro-Jørgensen, K. Erythropoietic activity and interferon production in LCM virus-infected nude mice. Acta path. microbiol. scand. Sect. B, 85 435–439 1977

The distinct haemopoietic lesions induced in ordinary mice during acute LCM virus infection are not mediated either by immunopathological mechanisms or by a direct cytopathogenetic effect of the virus. Mediation has recently been attributed to the high interferon activity found in mice with acute LCM virus infection. In this work, erythropoietic activity and interferon production have been studied in LCM virus-infected nude mice. Compared with ordinary mice, nude mice displayed a more moderate suppression of erythropoiesis and a very poor interferon response. Erythropoietic activity of thymus-transplanted nude mice was not significantly suppressed during the infection, and interferon responsiveness was not restored either by thymus-transplantation or by transfer of large numbers of spleen lymphocytes. The observations support the hypothesis that interferon plays a decisive role in the induction of haemopoietic disorders of mice with acute LCM virus infection. The puzzling question as to why nude mice and reconstituted nude mice are incapable of mounting a normal interferon response is discussed briefly

Key words: Erythropoietic activity; interferon production; LCM virus infection; nude mice.

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Received 29 77 Accepted 29 ii.77

Intraperitoneal (i.p.) inoculation of adult mice with lymphocytic choriomeningitis (LCM) virus causes no apparent clinical symptoms, but nevertheless induces profound immunologic and haemopoietic disorders, i.e. cell-mediated tumour immunity and allograft rejection are impaired (9 10) humoral immune responsiveness is markedly reduced (2, 3) and the post-irradiation formation of haemopoietic spleen colonies from pluripotent stem cells (CFU) is suppressed completely (1) It is conceivable that these immunologic and haemopoietic disorders may be causally interrelated (2, 3) In a recent study of haemopoietic precursor cells in LCM virus-infected mice, it was observed that both the number of CFU and *in vitro* colony-forming cells (CFC) and the erythropoietic activity (measured by incorporation of ^{59}Fe in blood and haemopoietic tissues) changed correspondingly during the infection (4) The virus inoculation led promptly to a uniform and profound suppression which was maintained for at least the first week of the infection. Then about day 10 after virus

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Acta path. microbiol. scand. Sect. B, 85: 435–439 1977

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The distinct haemopoietic lesions induced in ordinary mice during acute LCM virus infection are not mediated either by immunopathological mechanisms or by a direct cytopathogenetic effect of the virus. Mediation has recently been attributed to the high interferon activity found in mice with acute LCM virus infection. In this work, erythropoietic activity and interferon production has been studied in LCM virus-infected nude mice. Compared with ordinary mice, nude mice displayed a more moderate suppression of erythropoiesis and a very poor interferon response. Erythropoietic activity of thymus-transplanted nude mice was not significantly suppressed during the infection, and interferon responsiveness was not restored either by thymus-transplantation or by transfer of large numbers of spleen lymphocytes. The observations support the hypothesis that interferon plays a decisive role in the induction of haemopoietic disorders of mice with acute LCM virus infection. The puzzling question as to why nude mice and reconstituted nude mice are incapable of mounting a normal interferon response is discussed briefly.

Key words: Erythropoietic activity; interferon production; LCM virus infection; nude mice.

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Received 29.iv.77. Accepted 29.iv.77

Intraperitoneal (i.p.) inoculation of adult mice with lymphocytic choriomeningitis (LCM) virus causes no apparent clinical symptoms, but nevertheless induces profound immunologic and haemopoietic disorders, i.e. cell-mediated tumour immunity and allograft rejection are impaired (9, 10), humoral immune responsiveness is markedly reduced (2, 3), and the post-irradiation formation of haemopoietic spleen colonies from pluripotent stem cells (CFU) is suppressed completely (1). It is conceivable that these immunologic and haemopoietic disorders may be causally interrelated (2, 3). In a recent study of haemopoietic precursor cells in LCM virus-infected mice, it was observed that both the number of CFU and in vitro colony-forming cells (CFC) and the erythropoietic activity (measured by incorporation of ^{59}Fe in blood and haemopoietic tissues) changed correspondingly during the infection (4). The virus inoculation led promptly to a uniform and profound suppression which was maintained for at least the first week of the infection. Then about day 10 after virus

inoculation there was a marked restoration confined to the spleen and blood in which the stem cell number and the ^{59}Fe uptake reached supernormal values. The *transient* suppression was apparently not due either to any viral or immune-mediated cytopathic effect on haemopoietic cells, or to any virus-induced interference with the production of humoral stimulators of haemopoiesis. However with reference to studies on the non antiviral effects of interferon it was suggested that interferon might be the mediator of the suppression.

In a pilot investigation, using congenitally athymic nude mice (13) we observed that the endogenous spleen colony formation in nude mice treated with 500 R of X rays was not abolished during LCM virus infection. This gave rise to speculation as to whether the nude mice were elaborating a normal interferon response. Other workers (16) have found a normal interferon response in nude mice after administration of polyriboinosinic polyribocytidylic acid (poly I-C) and reduced interferon titres during Newcastle disease virus (NDV) infection. The latter observation is not surprising since NDV induction of interferon was also abolished in ordinary mice after treatment with anti lymphocyte serum (8). Since we know that interferon induction by LCM virus is radioresistant (4) and therefore probably not of lymphocytic origin nude mice could not readily be expected to be incapable of elaborating a normal interferon response during LCM virus infection.

In this work using the ^{59}Fe uptake in blood as a parameter of haemopoietic function we have performed some experiments with the aim of elucidating the following questions. 1) Is haemopoietic dysfunction induced in nude mice during LCM virus infection? 2) Do LCM virus infected nude mice elaborate the same amount of interferon as normal mice? 3) Does T cell reconstitution influence the haemopoietic function and/or the interferon response in LCM virus-infected nude mice?

MATERIALS AND METHODS

Virus. The LCM virus used was the Traub strain and the stock virus tissue culture fluid obtained after passage of the virus in monolayers of L cells (3). The stock virus was examined for possible contamination by Dr. *M. J. Collins* Jr., Microbiological Associates, Bethesda, Md., U.S.A. Except for LMC virus, none of the eleven murine viral contaminants described by *Collins & Parker* (7) were found, nor ectromelia and cytomegalovirus. Acute LCM virus infection was induced by intraperitoneal inoculation of 10^3 mean intracranial lethal doses (LD_{50}) of virus.

Mice. Nude mice of both sexes on a C3H background (C3H/nu/nu/10f/Bom) reared under specified pathogen-free conditions, were obtained from Gl. Bomholtgård, DK-8680 Ry and were in at least the sixth back-cross. They were kept under conventional conditions in filter top cages with humidified atmosphere and fed with autoclaved pills. The ordinary mice used in the experiments and for transplantation purposes belonged to a strictly inbred C3H strain (C3H/Ssd) raised and bred in this laboratory. All mice were aged 6 to 9 weeks at the time of infection, except the thymus-transplanted nude mice which were 14 weeks old.

Preparation of spleen cells. Pooled donor spleens were passed through stainless-steel meshes. Cells were washed twice in Hanks balanced salt solution, then counted and adjusted to 200×10^6 nucleated cells per ml. Nude mice received 1 ml of the cell suspension intraperitoneally.

Thymus transplantation. Whole thymuses were removed aseptically from newborn donors. Recipient nude mice aged 6 weeks were kept under slight propanidin anaesthesia (Epontol® 0.5 mg/gBW). Through an incision on the back, a thymus was placed subcutaneously on each side of the thorax. Grafted nude mice were used in the experiments two months later. There was a mortality of 35 per cent, most deaths occurring within the first month. Thymus grafts, lymph nodes and spleens were examined by microscopy to confirm the "take" of the thymuses and the development of histological characteristics of T cell reconstitution. No signs of graft versus host reaction were seen in any mouse.

Erythropoietic activity. For the measurement of the ^{59}Fe uptake amounts of 1.0 μCi ^{59}Fe (as ferric citrate sp.act. about 10 μCi/μg Fe Amersham England) in volumes of 0.5 ml were injected. After 24 hours, 100μl samples of blood were collected by cardiac puncture. The radioactivity of these samples was determined by means of a well type crystal gamma scintillation counter (Selektronik, Denmark). The percentages of injected iron present in the blood were then calculated assuming the total blood volume to amount to 5 per cent of the individual body weight.

TABLE 1 24 h ^{59}Fe Uptakes in Mice Injected with 1.0 µCi ^{59}Fe 4 or 12 Days after LCM Virus Inoculation

Group	Virus day	No. of mice	Mean ^{59}Fe 24 hour uptake in blood (% of control ± SD)
Ordinary mice (control)	—	4	100.0 ± 7.7
Ordinary mice	—4	4	3.2 ± 0.4
Nude mice	—	5	72.7 ± 25.4
Nude mice	—4	5	18.5 ± 7.1
Nude mice, thymus transplanted on day —60	—4	4	67.1 ± 28.1
Nude mice given 200 × 10⁶ spleen cells i.p. on day —5	—4	5	48.4 ± 7.7
Nude mice given 200 × 10⁶ spleen cells i.p. on day —25	—4	5	91.0 ± 30.1
Ordinary mice	—12	5	165.7 ± 14.9
Nude mice	—12	7	81.3 ± 28.1

Mean uptake of controls 30.1 %

Interferon assay. Interferon activity was determined by a plaque inhibition assay performed mainly as described by *Wagner et al.* (15). Briefly, serum or 20 per cent suspensions of homogenized spleens were dialyzed for 24 hours against saline adjusted with HCl to pH 2. After redialyzing to neutrality, aliquots of serial dilutions were added to duplicate monolayers of L cells grown in 50 mm Petri dishes. After incubation for 4 hours at 37° C the drained monolayers were infected with vesicular stomatitis virus adjusted to produce about 40 plaques, and then covered with agar medium and incubated for two days. The reciprocal of the dilution at which the number of plaques was reduced to 50 per cent of the control plaque count was considered to be the titre of interferon. The results of further attempts to characterize the interfering factor in the serum of acutely LCM virus-infected mice were reported in an earlier work from this laboratory (4).

RESULTS

Erythropoietic activity. Table 1 shows that both ordinary mice and nude mice displayed a reduced rate of erythropoiesis on day 4 after LCM virus inoculation. However, while the virus-induced reduction of the ^{59}Fe uptake in the ordinary mice was close to a factor 30 (from 100 to 3.2 per cent), the decrease that occurred in the nude mice was considerably less, viz. about 4-fold (from 72.7 to 18.5 per cent). On the other hand, on day 12 post infection, the ordinary mice showed evidence of a distinct compensatory increase which was absent in the nudes. It is of interest that the ^{59}Fe uptake in the blood of infected thymus-transplanted nude mice did not differ significantly from that of uninfected nudes. Likewise, the transplantation of nude mice with normal spleen cells was not associated with any enhancement of the virus-induced suppression of erythropoiesis. On the contrary, this caused a marked or complete resistance to the suppressive viral effect when performed either one day or three weeks before the virus inoculation, respectively. These rather surprising results were confirmed in an additional experiment with a similar design.

Interferon production. In this experiment, interferon titres were determined in groups of ordinary mice, nude mice, thymus-transplanted nude mice and nude mice which had been given 200 × 10⁶ spleen cells one day before virus inoculation. Pools of serum samples and spleens from groups of 4–5 mice were collected 48 hours after the LCM virus inoculation, since interferon titres in ordinary mice infected with LCM virus have been shown to reach peak levels at that time (4). Table 2 shows that interferon was present in appreciable titres in ordinary mice. In contrast, no or only threshold interfering activity

TABLE 2. *Interferon Activity 48 Hours after LCM Virus Inoculation*

Group	Virus day	No of mice	Interferon activity in serum	Interferon activity in spleen
Ordinary mice	—2	4	$2^{7.3}$	$2^{9.3}$
Nude mice	—2	4	$< 2^{3}$	$2^{4.3}$
Nude mice, thymus transplanted on day —62	—2	4	$< 2^{3}$	$2^{4.0}$
Nude mice given 200×10^{6} spleen cells on day —3	—2	4	$2^{3.4}$	$2^{4.4}$

could be detected in the sera from nude mice and reconstituted nude mice and the amount of interferon found in the spleen of these mice was consistently markedly reduced as compared to that found in the ordinary mice.

DISCUSSION

Though nude mice show no evidence of resistance to LCM virus multiplication (6) such mice were obviously less susceptible to LCM virus induced erythropoietic suppression than ordinary mice. This finding cannot be explained by the immunological unresponsiveness of nude mice to LCM virus infection (6) since haemopoietic dysfunction induced in ordinary mice is not mediated by immunopathological mechanisms (1) In our opinion the markedly reduced quantity of interferon found in nude mice offers a reasonable explanation, and adds further support to the hypothesis that interferon is the mediator of haemopoietic dysfunction provoked in LCM virus-infected mice (4)

Presumably the radioresistance of the interferon production induced by LCM virus (4) excludes the possibility that lymphocytes play any appreciable role as producers of the interferon. This assumption is supported by the fact that no significant restoration of interferon responsiveness took place in nude mice after thymus transplantation or transfer of spleen cells On the other hand these findings raise the very puzzling question as to why nude mice should be incapable of mounting a normal interferon response However to our knowledge it has not yet been documented convincingly that the absence of a functioning thymus epithelium with resulting T cell deprivation is the sole internal defect transmitted by the *nu* gene A second defect influencing the interferon responsiveness—at least to some inducers—can therefore not be excluded without further proof Alternatively differentiating stimuli originating in the foetal thymus might be necessary for the development of interferon responsiveness in related compartments, e.g the reticuloendothelial system. Such possible defects could not be expected to be restored either by transfer of large amounts of spleen cells or by grafting a neonatal thymus to adult nude mice. It is interesting that poly I-C *in nude mice* on a BALB/c background has been reported to induce normal or even augmented quantities of interferon (16) but the uncertainty of the precise histological localization of the cells responding to this inducer makes any conclusions premature.

The rather surprising observation of an almost unaffected erythropoiesis in LCM virus-infected thymus-transplanted nude mice and nude recipients of spleen cells may reflect a stimulatory effect of the T lymphocytes on haemopoiesis Thymus cells have been shown to exert a stimulatory effect on haemopoietic stem cells (11) and recently mediators derived from activated T lymphocytes, with the capacity to stimulate haemopoietic stem cells, have been described by *Cerny and coworkers* (5) We have evidence from pilot investigations that LCM virus-infected thymus-grafted nude mice produce almost normal titres of complement fixing antibodies and are able to eliminate the virus from the blood. Since

LCM virus elimination is brought about by activated T lymphocytes (12, 14). It is reasonable to assume that T cell-derived stimulators of haemopoiesis are in operation in the reconstituted nude mice and thus counteract the moderate suppression of erythropoiesis seen in untreated nude mice.

The hypothesis that interferon plays an important role with regard to the haemopoietic disorder provoked during LCM virus infection is supported by the findings presented here. From a pathogenetic point of view this may be of considerable interest when one considers the frequency with which haemopoietic and lymphoid disorders occur during many acute human and animal viral infections.

We wish to express our gratitude to Professor M. Volkert for valuable advice and helpful criticism during this work.

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Acta path. microbiol. scand. Sect. B, 85 440–448 1977

BACTERIA AND BACTERIAL ANTIGEN IN THE KIDNEY IN HUMAN CHRONIC RENAL DISEASE

Bacteriological and Immunofluorescence Studies

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Thomsen, O F & Olsen, T S Bacteria and bacterial antigen in the kidney in human chronic renal disease Bacteriological and immunofluorescence studies. Acta path. microbiol. scand. Sect. B 85 440–448 1977

Kidneys obtained by nephrectomy from 85 patients with chronic nephropathy were examined by bacterial culture and by immunofluorescence for a content of *E. coli* antigen. A panel of 10 *E coli* 0-antisera, *representing the strains most commonly causing urinary tract infection*, and antiserum against common enterobacterial antigen (CA) were used. Bacteria could be cultured from the nephrectomy specimens in 24 cases, mainly in cases of obstructive chronic pyelonephritis, analgesic nephropathy and congenital renal disease. By immunofluorescence type-specific 0-antigen was found in whole bacteria and amorphously in macrophages, CA only in whole bacteria. Whole bacteria could be visualized in 12 cases, macrophages only in two cases. Amorphous bacterial antigen was not observed outside phagocytizing cells. On the basis of these results, it seems unlikely that progression of the renal lesions in chronic renal disease is due to persistent bacterial antigen in the absence of viable bacteria. Chronic pyelonephritis, defined as an interstitial nephritis due to the effects of bacterial infection in the renal parenchyma and pelvic mucosa, appears always to be a secondary manifestation following obstruction or primary renal disease such as analgesic nephropathy or *congenital renal disease*

Key words Bacteria bacterial antigen kidney chronic nephropathy pyelonephritis immunofluorescence.

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Received 10.vii.77 Accepted 10 ii.77

In some types of human chronic renal disease thought to be due to bacterial infection of the renal pelvic mucosa and parenchyma and therefore diagnosed as chronic pyelonephritis, attempts to demonstrate micro-organisms in the renal tissue or in the urine have often failed (3 12, 17 18) Some investigators have suggested that following bacterial infection of the kidney residual bacterial antigen would remain at the site of inflammation also in the absence of viable bacteria, and it has been discussed whether persistent bacterial antigen could cause progressive chronic lesions of the tissue, possibly because of immunological mechanisms (3 12, 14) Immunofluorescence studies of *experimental* pyelonephritis seem

is lend support to this assumption, as it has been shown that bacterial antigen can persist in the renal tissue for varying periods, when the causative organisms can no longer be cultured from the tissue (7 20).

It was therefore of considerable interest that Aoki *et al.* 1969 (4) reported the finding by immunofluorescence of amorphous bacterial antigen in *human* renal tissue, not only from patients with enterobacteria in the urine and kidneys, but also in six out of nine cases of so-called chronic abacterial pyelonephritis, i.e. without demonstrable viable bacteria in the urine or renal tissue at the time of examination. The antigen, which was localized mainly in the interstitial tissue, was the common enterobacterial antigen (CA) shared by most strains of *E. coli* and other enterobacteria (16).

Although the immunofluorescence demonstration of CA in the renal tissue has been strongly questioned in subsequent investigations of experimental (22, 23) or human (21) pyelonephritis, the question of the possible role of persistent bacterial antigen is far from clarified.

The aim of this study was to examine if, and how often, persistent bacterial antigen occurs in chronically diseased human kidneys. The study was not limited to cases diagnosed as chronic pyelonephritis, because of the notoriously unspecific character of the histopathological lesions commonly regarded as indicating this disease, and because a secondary bacterial infection could conceivably occur in other types of chronic renal disease.

MATERIAL AND METHODS

Patients. Nephrectomy specimens from 85 consecutive patients with chronic advanced nephropathy were examined. The patients were 48 women and 37 men, whose ages ranged from 10 to 70 years, and averaged 40 years. Nine patients were under the age of 20 and 17 between 20 and 30 years. The material studied consisted of the original kidneys from 74 patients who had undergone bilateral nephrectomy in connection with renal transplantation, mostly less than three months after transplantation. Nephrectomy specimens from three patients who died without being transplanted, and from eight patients from a urological ward in whom unilateral nephrectomy had been performed were also included. From the time of transplantation immunosuppressive agents (azathioprine prednisone) were administered. For details concerning the therapy the reader is referred to (25).

Bacteriological technique. The kidneys were handled under sterile conditions, and specimens of tissue were removed for examination within half an hour after nephrectomy. For bacterial culture, tissue specimens of about 0.5–1.0 cm^3 were removed from the kidneys. The tissue specimens were cut with a pair of scissors, and the minced tissue was spread on blood-agar plates and added to brain-heart broth. All Gram-negative rods were identified according to standard procedures. In seven cases in which cultured *E. coli* strains had been preserved, these were type-tested by direct slide agglutination, both untreated and after heating to 100° C for 15 minutes, against the panel of *E. coli* 0-antisera mentioned below and against antiserum to *E. coli* 014 (anti-CA).

Immunofluorescence technique. For immunofluorescence examination specimens of kidney tissue were quick-frozen at −70 C in an embedding medium for frozen tissue specimens ("tissue-tek" Miles laboratories Inc.) (at the beginning of the study some kidneys were frozen in isopentane). The specimens were stored at 70° C until required. The remaining kidney tissue was fixed in 10 per cent formalin and prepared for conventional light microscopy. In the indirect immunofluorescence method, the first layer applied was an unlabelled rabbit antiserum against *E. coli* 0-antigen, and the second layer was a commercially available fluorescein isothiocyanate-labelled antiserum against rabbit immunoglobulin (FITC-labelled goat anti-rabbit-γ globulin, Behringwerke). By immuno-electrophoresis, this conjugate revealed a single, strong precipitation line corresponding to rabbit IgG. The molar F/P ratio was 1.8.

As *E. coli* is the commonest urinary pathogen (11) a panel of various antisera against *E. coli* 0-antigen was applied as the first layer in the indirect immunofluorescence staining. This panel included the following 0-serotypes most frequently associated with urinary tract infection: 01, 02, 04, 06, 07, 08, 09, 018, 022 and 075. Moreover anti-*E. coli* 014 was used representing antibody against CA. The antisera as well as the homologous bacteria were kindly supplied by Dr. *F. Ørskov*, The International Escherichia Centre, Statens Seruminstitut, Copenhagen.

The *E. coli*-antisera were used in 1:10 dilutions and the FITC-labelled goat anti-rabbit immunoglobulin in a 1:20 dilution. The proper dilutions were determined in immunofluorescence-titration experiments on rat kidneys with experimental pyelonephritis, evoked by homologous strains of *E. coli* bacteria.

Sectioning staining specificity controls and microscopic examination were carried out as previously described (22, 23) The immunofluorescence examinations were carried out without any knowledge of the histo-pathological diagnoses.

Histochemical technique Cryostat-cut frozen sections, consecutive to those stained for immunofluorescence were stained for acid phosphatase by the method of *Burstone* as previously described (24)

Morphological diagnostic criteria. Paraffin sections stained with hematoxylin-eosin, van Gieson and periodic acid-Schiff (PAS) were examined. The pathological diagnoses were based on the nephrectomy specimens and in several cases, also on previous renal biopsies.

The following diagnostic criteria were applied

Obstructive chronic pyelonephritis Kidneys with severe interstitial and pelvic mucosal infiltration with mononuclears, interstitial fibrosis and leucocyte casts. Obstruction or dysfunction of the urinary tract was a prerequisite for this diagnosis (urinary calculi, congenital stenosis, severe primary reflux, etc.)

Analgesic nephropathy Chronic interstitial nephritis associated with multiple papillary necroses. All patients with this renal lesion had a history of excessive analgesic ingestion

Congenital renal disease Bilateral polycystic renal disease, five cases lobar hypoplasia (Ask Upmark) eight cases medullary cystic disease one case and other cystic anomalies, three cases. Chronic interstitial nephritis was present in the areas between the cysts.

Glomerulonephritis Chronic renal disease with inflammatory glomerular lesions (hypercellularity many remnants of crescents, glomerulo-capsular adhesions) still discernible in spite of advanced glomerular sclerosis. Among the 13 patients with this diagnosis one or more biopsy specimens had been obtained in earlier stages of the renal disease in 10 and these biopsies corroborated the diagnosis. *In eight patients, glomerular immunodeposits* were demonstrated.

Nephrosclerosis Chronic renal disease with arteriolosclerosis and/or arteriolonecrosis. All patients in this category had sustained hypertension.

Diabetic nephropathy Advanced nodular glomerulosclerosis in patients suffering from long-term diabetes.

Unclassifiable chronic nephropathy *These* patients had severe interstitial fibrosis with nephronic atrophy and lymphocytic infiltrates. The following conditions were *not* present papillary necroses, urinary tract obstruction, glomerulitis or glomerular immunodeposits by immunofluorescence diabetes or severe sustained hypertension. The category is probably partly identical with what has sometimes been called chronic non-obstructive pyelonephritis.

Other diagnoses Chronic renal failure referable *to sequelae of bilateral* cortical necrosis was present in one patient, and renal atrophy caused by bilateral arterial stenosis in another

Statistical analysis. The χ^2 test or Fisher's exact test was used in the evaluation of the incidence of viable *bacteria in* the nephrectomy specimens in the patho-anatomical groups. A p value or a 2 p value, respectively below 0.05 was considered to indicate statistical significance.

RESULTS

Bacterial culture Such cultures were carried *out from the nephrectomy specimens* in 79 out of the 85 patients (Table 1) Bacteria were obtained in 24 cases (30.4 per cent) viz. *E coli* in 14 and staphylococci, Proteus, *Pseudomonas aeruginosa* Klebsiella or *E cloacae* in 10 cases. Bacteria in the nephrectomy specimens occurred with a significantly higher incidence in the 43 patients with obstructive chronic pyelonephritis, analgesic nephropathy or congenital renal disease than in the 36 patients with other renal diseases ($p < 0.0005$) No significant difference was disclosed between the incidence of bacteria in patients with pyelonephritis and patient with analgesic nephropathy ($2\ p = 0.321$) or in those with congenital disease ($2\ p = 0.084$)

Immunofluorescence examination The bacterial antigen occurred in two forms as whole bacteria recognizable by their forms, and as amorphous antigen phagocytized by polymorphonuclear leucocytes or macrophages. Using antiserum against CA *whole* bacteria were seen in the kidneys of 12 patients, including seven in whom the bacteria were also seen in stains with *E coli* 0-antisera (Tables 1 and 2) The bacteria occurred very sparsely either isolated or in small groups Most frequently they were *seen in* the pelvic-calyceal exudate (Fig 1) among *polymorphonuclear leucocytes, in* or beneath the mucosal epithelium (Fig 2) in the collecting tubules near the mucosa and in inflamed cystic lumina and walls. They did not occur in glomeruli or in relation to blood vessels.

TABLE 1 *The Results of Bacterial Culture from the Nephrectomy Specimens and Immunofluorescence Finding of Bacterial Antigen in the Kidneys of 85 Patients*

Histo-anatomical diagnoses	No. of patients	Bacterial culture from nephrectomy specimens			Immunofluorescence			
			Growth of		*E. coli* O-antigen in		CA in	
		carried out in	*E. coli*	other bact.	whole bact.	amorph. in macroph.	whole bact.	amorph. in macroph.
Obstructive chronic pyelonephritis	11	10	2	6	1		1	
Analgesic nephropathy	20	17	8	1	2		5	
Congenital renal disease	17	16	4	2	4	2	5	
Glomerulonephritis	13	12		1			1	
Nephrosclerosis	5	5						
Diabetic nephropathy	3	3						
Unclassifiable chronic nephropathy	14	14						
Other diagnoses	2	2						
Total	85	79	14	10	7	2	12	

Amorphous bacterial antigen in polymorphonuclear leucocytes was present in the same localizations as whole bacteria. In addition, in two cases of congenital cystic disease large amounts of amorphous bacterial antigen occurred intracellularly as phagocytized by macrophages (Figs. 3 and 4). From both, *E. coli* could be cultured, which in direct bacterial agglutination tests appeared to be the serotypes 075 and 07 respectively and the corresponding 0-antisera yielded a positive immunofluorescence reaction, demonstrating bacterial antigen in the renal macrophages. The fluorescence reaction was negative with the other *E. coli* 0-antisera and with antiserum against CA.

In none of the cases, free non phagocytized bacterial antigen was seen in the interstitial tissue or elsewhere.

Comparing the results of bacterial cultures with the immunofluorescence findings (Table 2) it appears that CA could be demonstrated in whole bacteria in nine cases infected with *E. coli* in two cases infected with Proteus and *E. cloacae* respectively and in one case in which bacterial culture had not been performed. With the various *E. coli* 0-antisera, homologous 0-antisera was seen in 7 out of the 14 cases infected with *E. coli* in an additional case, equivocal fluorescence was seen. In seven cases, direct bacterial agglutination tests were carried out on the cultured bacteria, using the same antisera as in the immunofluorescence examinations. These revealed positive results, and in the five cases with positive reaction of both agglutination and immunofluorescence, these two methods showed accordance as to the demonstration of the 0-serotype.

Bacterial antigen was not demonstrated by immunofluorescence in kidneys from which bacterial culture had been negative.

Histochemical examination. Renal tissue from the 85 patients was stained histochemically in order to visualize active macrophages on the basis of acid phosphatase-positive cytoplasmic granules, corresponding to what has been observed in experimental rat pyelonephritis (24). Numerous interstitial macrophage-like cells occurred in the tissue in three cases. Two of these were the same as those in which bacterial antigen-containing macrophages could be demonstrated by immunofluorescence and the histochemically

TABLE 2. *The 25 Patients in whom Bacterial Growth from the Nephrectomy Specimens and/or Immunofluorescence Findings were Positive*

Patients	Patho-anatomical diagnoses	Bacterial growth from nephrectomy specimens*	Immunofluorescence with *E coli* O-antisera: O1	O2	O4	O6	O7	O8	O9	O18	O22	O75	(CA) O14	O-serotype by direct agglutination
CA	Obstructive chronic pyelonephritis	+ + *E coli*				+							+	O6
EC	—	+ + *E coli*												O8
AB	—	+ *Pseudom aer*												
LH	—	+ + *Pseudom aer*												
HH	—	+ *Staph. aur*												
JO	—	+ *Staph. alb*												
EB	—	+ Proteus												
BJ	—	+ *Proteus Morg*												
LA	Analgesic nephropathy	+ + *E. coli*						(+)						
MC	—	+ + *E coli*											+	O8
GB	—	+ *E. coli*												
ER	—	+ + *E. coli*												
JS	—	+ + *E coli*												
SP	—	+ + *E coli*											+	
RS	—	+ *E. coli*		+									+	
NC	—	+ + *E. coli*									+		+	O22
MH	—	+ *Klebsiella pneum*												
BM	—	Not tested											+	
EM	Congenital renal disease	+ *E. coli*			+								+	
CJ	—	+ *E. coli*				+							+	O6
FV	—	+ + *E coli*					+						+	O7
JH	—	+ *E. coli*										+	+	O75
HW	—	+ + *Klebsiella pneum*												
TR	—	+ + *Proteus Morg*											+	
ED	Glomerulonephritis	+ + *E. cloacae*											+	

\+ = < massive growth
\+ + massive growth

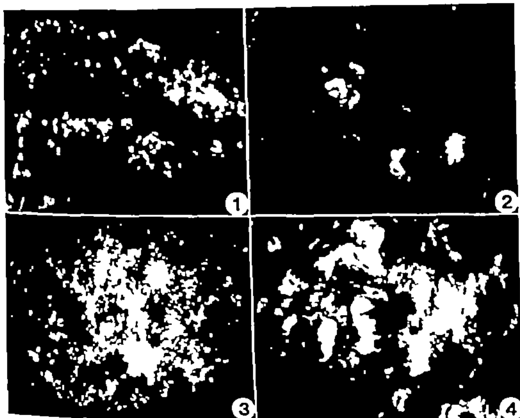


Fig. 1. Pelvic exudate with fluorescent bacteria, partly clumping. Section stained with anti-CA serum. × 300

Fig. 2. Clumps of bacteria beneath pelvic epithelium. Section stained with anti-07 serum. × 300

Fig. 3. Interstitial fluorescent macrophages containing amorphous bacterial antigen. Section stained with anti-07 serum. × 120.

Fig. 4. Group of fluorescent macrophages containing amorphous bacterial antigen. Section stained with anti-075 serum. × 480

stainable cells were localized in the same places as the actively phagocytizing macrophages (Figs. 5 and 6). The third case was one of obstructive chronic pyelonephritis. Here the cells were localized in the ulcerated regions of inflamed dilated calyces. From these nephrectomy specimens, *Pseudomonas aeruginosa* could be cultured, which could not be visualized by the panel of *E. coli* antisera or with anti-CA serum.

Sporadically and in smaller numbers, groups of interstitial acid phosphatase-positive cells were present in the kidneys of seven other patients. In six of these whole bacteria could be seen by immunofluorescence fundamentally with the same localizations as the cells.

DISCUSSION

Our results showed that viable bacteria in the kidneys were nearly exclusively present in patients suffering from obstructive chronic pyelonephritis, analgesic nephropathy and congenital renal disease. Other authors have revealed bacteria in the renal tissue with various frequency. *Jacobson & Newman* (14)

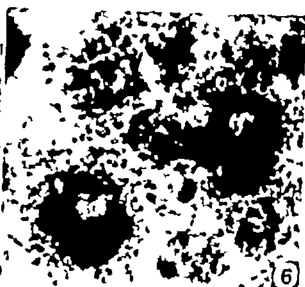



Fig 5 Interstitial acid phosphatase-positive cells (presumably macrophages) infiltrating the surroundings of a tubule Section stained for acid phosphatase. × 120

Fig 6 Two cells with abundant acid phosphatase-positive cytoplasmic granules (presumably macrophages) Section stained for acid phosphatase × 1200

in a biopsy material of *chronic pyelonephritis* found 7 7 per cent with positive bacterial growth in a similar material *Brun et al* (5) observed 30 4 per cent with bacteria and in an autopsy study of chronic pyelonephritis *Pawlowski et al* (18) found about 18 per cent with positive bacterial growth. These three studies included chronic pyelonephritis in a wider sense of the term without differentiation between obstructive and non-obstructive forms In the present study the term pyelonephritis was restricted to cases with interstitial nephritis associated with a documented obstruction and in this diagnostic group we found that eight of the 10 investigated patients had viable bacteria in their renal specimens On the other hand it is noteworthy that none of our patients with *chronic unclassifiable nephropathy* did have positive bacterial cultures. In these kidneys, interstitial lymphocytic infiltration was invariably present and cases of this type are no doubt frequent among patients usually diagnosed as having a chronic non-obstructive pyelonephritis Since moreover bacterial antigen was never found in these kidneys, we are of the opinion that there is no available evidence of bacterial aetiology as responsible for the chronic nephropathy of this category

The frequent finding of bacteria in the nephrectomy specimens from the patients with *analgesic nephropathy* presumably reflects a susceptibility to secondary bacterial infection, as shown experimentally (9) *Clausen & Jensen* (6) in a study of renal biopsies from 30 patients with a high analgesic intake found sterile renal tissue in all cases. The positive results of the present study performed on tissue from patients with terminal renal failure, indicates that bacterial infection is a late complication of analgesic renal disease, as also maintained by *Cloor* (10) In patients with *congenital renal disease* the frequent occurrence of bacteria in the kidney tissue probably also reflects the tendency to secondary bacterial infection known to develop in this category of patients (13)

If chronic pyelonephritis is defined as a disease characterized by chronic inflammation of the pelvic mucosa and the renal parenchyma due to bacteria present in the kidney (8) this disease appears always to be a secondary manifestation either in ob-

struction of the urinary tract or in primary diseases of the renal parenchyma, such as analgesic nephropathy with papillary necrosis or congenital renal disease.

Bacterial antigen was not revealed by immunofluorescence in all specimens with viable bacteria, and antigen was never demonstrated in specimens from which a bacterial growth culture had been negative. There may be several explanations of the apparently greater sensitivity of the growth culture in the disclosure of infection. First, 10 of the patients were infected with bacteria other than *E. coli*, which could therefore not be visualized by our panel of 0-antisera. Two of the 10 (one case with Proteus and one with *E. cloacae*) could be demonstrated on the basis of a content of CA, whereas others (staphylococci, Pseudomonas) do not contain CA. Secondly not all cases infected with *E. coli* could be detected with the panel of 0-antisera. This might be expected because the panel of antisera did not cover all types of *E. coli* occurring in the human kidney. However the use of these antisera was based on the findings of several investigators, showing that the corresponding, relatively few 0-serotypes of *E. coli* are responsible for from one half up to two thirds of *E. coli* urinary tract infections (2, 11, 19, 26, 27). Finally it should be mentioned that, for example, studies of tubercle bacilli in sputum indicate that not all tubercle bacilli in a preparation are revealed by fluorescence microscopy and that a higher proportion of positive results is obtained by culture (28); the same is probably true of immunofluorescence investigations of tissue specimens.

The principal aim of this study was to investigate whether persistent bacterial antigen could be responsible for progressive chronic renal disease in cases in which no viable bacteria could be detected in the kidneys.

Compared with other immunofluorescence studies, the present results are in disagreement with those of *Aoki et al.* (4) who in cases of abacterial chronic pyelonephritis demonstrated amorphous bacterial antigen by using antiserum against CA. On the other hand, our results are consistent with those of *Schwartz & Cotran* (21) who demonstrated CA in only one out of nine patients with chronic pyelonephritis, but in three out of five with acute pyelonephritis. *Kaijser et al.* (15) in their study of 14 children with recurrent pyelonephritis by means of a panel of *E. coli*-antisera, found bacterial antigen in only one, whereas CA could not be demonstrated at all. So the use of antiserum against CA in immunofluorescence microscopy may seem to be restricted to the demonstration of fresh bacterial antigen, i.e. mainly whole bacterial forms.

A loss of bacterial antigenicity may be due to the formation of protoplast forms, known to occur in the renal interstitium under certain conditions (1). Bacterial antigen may also become catabolized fairly early after an infection, a mechanism probably operative in experimental pyelonephritis (22, 23). Active macrophages (visualized in this study by positive staining for acid phosphatase) are probably of importance in this process. Based on these considerations, it seems unlikely that bacterial antigen can persist in the renal tissue for prolonged periods after the disappearance of the bacteria present in an initial phase of the disease.

Our thanks is due to Dr. *Tage Hjort*, Institute of Medical Microbiology, University of Aarhus, for his valuable advice and help during the study.

This study was supported by a grant from the *Danish Medical Research Council*.

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Acta path. microbiol. scand. Sect. B, 85: 449–454, 1977

BACTERIURIA AND RENAL INFECTION IN KIDNEY-TRANSPLANT RECIPIENTS

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Thomsen, O. F. & Hansen, H. E. Bacteriuria and renal infection in kidney-transplant recipients. Acta path. microbiol. scand. Sect. B, 85: 449–454, 1977.

In 64 patients who had undergone renal transplantation, later on followed by bilateral nephrectomy, bacterial growth culture was performed from the original kidneys. The presence of bacteria in the nephrectomy specimens was compared with the occurrence of significant bacteriuria before transplantation and in the period between transplantation and nephrectomy. Bacteria could be cultured from the nephrectomy specimens of 18 (28.1 per cent) of the patients, almost exclusively confined to cases of obstructive chronic pyelonephritis, analgesic nephropathy and congenital renal disease. Before transplantation, bacteriuria had been recorded in 54.4 per cent of the patients, most frequently in the three groups of diseases just mentioned. Between the transplantation and nephrectomy, bacteriuria occurred in 75.0 per cent of the patients. Patients with E. coliuria before transplantation were particularly liable to have E. coliuria also after the transplantation and to E. coli in the nephrectomy specimens, whereas patients in whom E. coliuria did not occur until in the post-transplantation period were less susceptible to E. coli infection involving the kidneys. Probably the presence of bacteria in the nephrectomy specimens is related to the primary disease rather than to immunosuppressive and antibiotic agents administered in the post-transplantation period.

Key words: Bacteriuria; urinary tract infection; pyelonephritis; kidney transplantation.

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Received 10. .77. Accepted 10.vii.77

In patients with chronic nephropathy, most of them kidney-transplant recipients, it has been demonstrated that specimens from the original kidneys obtained at nephrectomy contained bacteria in about 30 per cent of all patients (12). This was found in cases of obstructive chronic pyelonephritis, analgesic nephropathy and congenital renal disease, but only exceptionally in other renal disorders.

As bilateral nephrectomy was usually performed some time after transplantation, it is clear that the frequency with which bacteria could be demonstrated in the nephrectomy specimens may be influenced by immunosuppressive and/or antibiotic agents administered in the period between transplantation and nephrectomy. On the other hand, the occurrence of bacteria in the nephrectomy specimens may also primarily be associated with the original renal disease. On this background, the aim of the present study was to evaluate the presence of bacteria in the nephrectomy specimens in relation to the occurrence of urinary tract infection (as assessed by bacteriuria) before transplantation and in the period between transplanta-

tion and nephrectomy in patients with various kidney diseases.

MATERIAL AND METHODS

Patients The original kidneys from 64 consecutive patients who had received a renal allograft were examined. The patients were 39 women and 25 men whose ages ranged from 10 to 63 years and averaged 39 years. Bilateral nephrectomy was, on an average carried out less than three *months after* transplantation. Nine patients were transplanted more than once because of rejection of the graft. In these patients, bacteriuria before the first transplantation was registered, and after the last, successful transplantation, in relation to which bilateral nephrectomy was done The patients in this series were included in an immunofluorescence study on bacterial antigen reported elsewhere in this issue (12) The present study comprised the transplant recipients in whom nephrectomy was performed subsequent to transplantation and from whose nephrectomy specimens a bacterial growth culture had been carried out.

Therapy The immunosuppressive agents administered were azathioprine and prednisone. Immediately before transplantation azathioprine was given in a single dose of 5 mg/kg body weight and thereafter 1–3 mg/kg body weight/day depending on renal function and the number of white blood cells. Prednisone treatment was initiated 1–2 days after transplantation with a dosis of 2–2.5 mg/kg/day In the course of 3–4 weeks, the dosis was reduced to 30–40 mg/day and during the following year to 10–15 mg/day Antibiotics were not given routinely to patients with significant bacteriuria. During the period before nephrectomy the guide lines were as follows In the presence of clinical signs of urinary tract infection and significant bacteriuria, antibiotics were given according to the results of sensitivity test. Streptomycin, ampicillin and a combination of trimetroprime and sulphonamides were the most commonly used antibiotics If urinary tract infection was present, bilateral nephrectomy was carried out as soon as the graft function had reached a stable level.

Bacteriology Bacteriological examination of the nephrectomy specimens was carried out according to the technique described in (12) From these quantitative bacteriological studies were not performed.

In all patients, the urine—according to standard procedures—was examined for bacteria twice weekly just before transplantation and between transplantation and nephrectomy Significant bacteriuria was defined as the presence of ≧ 10^5 bacteria per ml of voided midstream urine. The clinical records of the patients were searched for evidence of significant bacteriuria before transplantation and in the period between transplantation and nephrectomy

Pathology In light microscopy paraffin sections stained with hematoxylin-eosin, van Gieson and periodic acid-Schiff (PAS) were examined. Pathoanatomical examination of the original kidneys (based both on the nephrectomy specimens and previous renal biopsies) resulted in eight main categories of advanced renal lesions. The diagnostic criteria used for this classification were as described in (12)

Statistical analysis Fisher's exact test or the χ^2 test was used. A 2 p value or a p value below 0.05 respectively was considered to indicate statistical significance.

RESULTS

Table 1 shows the occurrence of significant bacteriuria and bacterial growth from the nephrectomy specimens in the eight groups of patients. Before transplantation (T) bacteriuria was present in 22 patients (34.4 per cent) Patients with obstructive chronic pyelonephritis, analgesic nephropathy and congenital renal disease predominated with 17 patients, while patients with chronic glomerulonephritis had sterile urine before T Several of these patients had also bacteriuria between T and nephrectomy (N) Bacteriuria only between T and N occurred in all groups (except the two smallest) with a frequency of 33–73 per cent. Almost half of the patients with unclassifiable nephropathy and one third of patients with glomerulonephritis, did not have bacteriuria at all.

Among the 64 patients, bacteria could be cultured from the nephrectomy specimens in 18 (28.1 per cent) 17 of these patients belonged to the three groups with a high incidence of bacteriuria before T mentioned above.

The bacteriological data of the 18 patients with bacteria in the nephrectomy specimens appear from Table 2 Bacteriuria between T and N occurred in 17 patients, including 15 in whom the same bacterial species was present in the urine and nephrectomy specimens. Among the latter 15 eight had bacteriuria before T all of them again with the same bacterial species.

TABLE 1 *Patho-anatomical Lesions Bacteriuria and the Occurrenc of Bacteria in the Nephrectomy Specimens from 64 Kid ey-Tran plant Recipients*

Patho-anatomical diagnoses	No. of patients	Bacteriuria				Bacteria in nephr specimens
		Only before transpl.	Before transpl. and between transpl. and nephr	Only between transpl. and nephr	Absent	
Obstructh chronic pyelonephritis	6		3	2	1	5
Analgesic nephropathy	16		10	6		8
Congenital renal disease	13	2	2	7	2	4
Glomerulonephritis	11			8	3	1
Nephrosclerosis	3			2	1	
Diabetic nephropathy	3		1		2	
Unclassifiable chronic nephropathy	11	1	2	4	4	
Sequelae of cortical necrosis	1		1			
Total	64	3	19	29	13	18

TABLE 2 *Bacteriuria in th 18 Patients with Bacteria in the Neph ectomy Specimens*

Patient	Patho-anatomical diagnoses	Bacteria cultured from nephrectomy specimens)	Bacteriuria between transpl. and nephr	Bacteriuria before transpl.
CA	Obstructi chronic pyelonephritis	+ + *E. coli*	—	—
EG	—	+ + *E. coli*	*E. coli*	—
AB	—	+ *Pseudom aer*	*Pseudom aer*	—
HH	—	+ *St ph. ar*	*E. oli*	Pseudom., *B. anitratum*
BJ	—	+ Proteus	Proteus, Pseudom	Proteus, Pseudom
LA	Analgesic nephropathy	+ + *E. coli*	*E. coli*	*E. coli* Klebsiella
MC	—	+ + *E. oli*	*E. coli*	*E. coli*
ER	—	+ + *E. coli*	*E. oli*	*E. coli*
JS	—	+ + *E. oli*	*E. oli*	*E. coli*
SP	—	+ + *E. coli*	*E. oli*	*E. coli*
RS	—	+ *E. oli*	*E. coli*	*E. coli*
KC	—	+ + *E. coli*	*E. coli*	—
MH	—	+ Klebsiella	Klebsiella	—
EM	Congenital renal disease	+ *E. coli*	*E. coli*	*E. coli*
GJ	—	+ *E. coli*	*E. coli*	—
JH	—	+ *E. coli*	*E oli*	—
EA	—	+ + *E. oli*	*E coli*	—
ED	Glomerulonephritis	+ + *E. loacae*	Klebsiella	—

= < mass growth
= mass growth

Table 3 shows that bacteriuria had been present at some time in 17 (94.4 per cent) of the 18 patients with bacteria in the renal specimens and in 34 (73.9 per cent) of the 46 patients with sterile nephrectomy specimens this difference is not significant (2 p > 0.1) Bacteriuria before T had been recorded in nine patients (50.0 per cent) with

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MATERIAL AND METHODS

Patients The original kidneys from 64 consecutive patients who had received a renal allograft were examined The patients were 39 women and 25 men whose ages ranged from 10 to 63 years and averaged 39 years. Bilateral nephrectomy was, on an average carried out less than three months after transplantation. Nine patients were transplanted more than once because of rejection of the graft. In these patients, bacteriuria before the first transplantation was registered and after the last, successful transplantation in relation to which bilateral nephrectomy was done The patients in this series were included in an immunofluorescence study on bacterial antigen reported elsewhere in this issue (12) The present study comprised the transplant recipients in whom nephrectomy was performed subsequent to transplantation and from whose nephrectomy specimens a bacterial growth culture had been carried out

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Among the 64 patients, bacteria could be cultured from the nephrectomy specimens in 18 (28 1 per cent) 17 of these patients belonged to the three groups with a high incidence of bacteriuria before T mentioned above.

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TABLE 1 *Patho-anatomical Lesions, Bacteriuria and the Occurrence of Bacteria in the Nephrectomy Specimens from 64 Kidney-Transplant Recipients*

Patho-anatomical diagnoses	No. of patients	Bacteriuria				Bacteria in nephr specimens
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Analgesic nephropathy	16		10	6		8
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Glomerulonephritis	11			8	3	1
Nephrosclerosis	3			2	1	
Diabetic nephropathy	3		1		2	
Unclassifiable chronic nephropathy	11	1	2	4	4	
Sequelae of cortical necrosis	1		1			
Total	64	3	19	29	13	18

TABLE 2. *Bacteriuria in the 18 Patients with Bacteria in the Nephrectomy Specimens*

Patient	Patho-anatomical diagnoses	Bacteria cultured from nephrectomy specimens*)	Bacteriuria between transpl. and nephr	Bacteriuria before transpl.
CA	Obstructive chronic pyelonephritis	++ *E. coli*	—	—
EC	—	++ *E. coli*	*E. coli*	—
AB	—	+ *Pseudom. aer.*	*Pseudom. aer.*	—
HH	—	+ *Staph. aur.*	*E. coli*	Pseudom., *B. [illegible]*
BJ	—	+ Proteus	Proteus, Pseudom.	Proteus, Pseudom.
LA	Analgesic nephropathy	++ *E. coli*	*E. coli*	*E. coli*, Klebsiella
MC	—	++ *E. coli*	*E. coli*	*E. coli*
ER	—	++ *E. coli*	*E. coli*	*E. coli*
JS	—	++ *E. coli*	*E. coli*	*E. coli*
SP	—	++ *E. coli*	*E. coli*	*E. coli*
RS	—	+ *E. coli*	*E. coli*	*E. coli*
KC	—	++ *E. coli*	*E. coli*	—
MH	—	+ Klebsiella	Klebsiella	—
EM	Congenital renal disease	+ *E. coli*	*E. coli*	*E. coli*
GJ	—	+ *E. coli*	*E. coli*	—
JH	—	+ *E. coli*	*E. coli*	—
EV	—	++ *E. coli*	*E. coli*	—
ED	Glomerulonephritis	++ *E. cloacae*	Klebsiella	—

= < massive growth
= massive growth

Table 3 shows that bacteriuria had been present at some time in 17 (94.4 per cent) of the 18 patients with bacteria in the renal specimens and in 34 (73.9 per cent) of the 46 patients with sterile nephrectomy specimens; this difference is not significant (2 p > 0.1). Bacteriuria before T had been recorded in nine patients (50.0 per cent) with

TABLE 3 *The Relation between Bacteriuria and the Occurrence of Bacteria in the Nephrectomy Specimens*

	No of patients	Bacteriuria		
		Present before transpl.	Only between transpl. and nephr	Absent
Bacteria in the nephrectomy specimens	18	9	8	1
Sterile nephrectomy specimens	46	13	21	12
Total	64	22	?9	13

TABLE 4 *The Relationship between Bacteria in the Nephrectomy Specimens and Bacteriuria*

Bacteria in neph rectomy specimens	No of patients	E. coliuria		Other bacteriuria		No. bacteri uria
		Before transpl	Only between transpl. and nephr	Before transpl.	Only between transpl and nephr	
E coli	13	7	5			1
Others	5	1		1	3	
Absent	46	5	11	8	10	12
Total	64	13	16	9	13	13

bacteria in the nephrectomy specimens, and in 13 (28.3 per cent) with sterile specimens this *difference is not significant* ($2\ p > 0\ 1$) However when the bacterial species is con sidered (Table 4) it appears that E. coliuria before T had been present in seven (53.9 per cent) of the 13 patients with *E coli* in the *nephrectomy specimens,* but only *in six* (11 8 per cent) of the 51 patients with other bacteria or with sterile nephrectomy speci mens, i.e. E coliuria before T was signif icantly *more frequent in the former group* of patients ($2\ p = 0\ 005$) In contrast E. coliuria only between T and N did not occur significantly more frequently in pa tients with *E coli* in the nephrectomy spec *imens than in those* with other bacteria or with sterile nephrectomy *specimens* ($2\ p > 0\ 2$)

Between T and N bacteriuria was present in 48 patients (*75 0 per cent*) Table 5 shows that of the 22 patients with bacteriuria be fore T 19 (86 4 per cent) had bacteriuria *also between T and N and of the 42 patients* without previous bacteriuria, 29 (69 0 per cent) had bacteriuria between T and N This difference is not significant ($p > 0.2$) How ever once more considering the bacterial species, it appears that out of the 13 patients with E. coliuria before T 11 (84 6 per cent) had E. coliuria also between T and N *whereas, of the* 51 patients with other or no *bacteriuria before T* only *20* (*39 2 per cent*) had E coliuria between T and N i e this occurrence was significantly more frequent in the former group of patients ($p < 0\ 01$)

Table 6 *shows that among the* 48 patients with bacteriuria between T and N antibiotic treatment was given to 18 with clinical signs of urinary tract infection and to seven with other infection e g *pneumonia or wound* infection Bacteria were found in the neph

rectomy specimens from nine of the 18 patients to whom antibiotics had been given because of urinary tract infection. Seven of the 23 bacteriuric patients who had not received antibiotics had also bacteria in the nephrectomy specimens, i.e. no significant difference in the occurrence of renal bacteria was found in these two groups of patients (2 p > 0.2)

TABLE 5. *The Relation between Bacteriuria before Transplantation and between Transplantation and Nephrectomy*

Bacteriuria before transpl.	No. of patients	Bacteriuria between transpl. and nephr. E. coli uria	Other	Absent
E. coliuria	13	11	1	1
Other bacteriuria	9	4	3	2
Absent	42	16	13	13
Total	64	31	17	16

TABLE 6. *Antibiotic Treatment and Bacteria in the Nephrectomy Specimens in the 48 Patients with Bacteriuria between Transplantation and Nephrectomy*

	No. of patients with bacteriuria between transpl. and nephr.	No. of patients with bacteria in nephr. specimens
Antibiotics because of urinary tract infection	18	9
Antibiotics because of other infection	7	1
No antibiotics	23	7
Total	48	17

DISCUSSION

The immunosuppressive therapy given to most of the patients during the period before nephrectomy might have increased the frequency with which bacteria invaded the renal parenchyma, because of a lowered resistance in the patients against bacterial infection as a secondary effect of the therapy. If however parenchymal infection had usually developed from this urinary tract infection, bacteria in the nephrectomy specimens should be expected to occur with the same frequency in the various diagnostic groups. That this was not the case speaks strongly against the hypothesis mentioned above. We therefore consider a positive bacterial culture from the nephrectomy specimens as an indication of renal parenchymal infection not obviously related to circumstances following transplantation. This is also supported by the fact that bacteriuria *before* transplantation was most often recorded in the same diagnostic groups as those with a high frequency of bacteria in the kidneys: obstructive chronic pyelonephritis, analgesic nephropathy and congenital renal disease. No difference in the frequency of infected nephrectomy specimens could be recorded in bacteriuric patients who had or had not received antibiotic treatment. Thus, specific antibiotic therapy did not seem to have eradicated the bacteria from the kidneys, which shows the justice of our practice in removing the original kidneys as soon as possible after transplantation. Probably the presence of bacteria in the nephrectomy specimens should primarily be associated with the tendency to bacterial infection in the original renal disease.

The patterns found for bacteriuria in relation to the time of transplantation and to bacterial renal infection were as follows. Patients with E. coliuria before transplantation were particularly liable to have E. coliuria also between transplantation and nephrectomy and, moreover *E. coli* infection involving the kidneys. In many cases, the same bacterial species as in the nephrectomy specimens was found in the urine not only after but also before the transplantation. Although typing of the bacteria was not performed it may be assumed that this indicates a high susceptibility to latent or recurrent *E. coli* infection in these patients. In comparison, *Leigh* (5) found that patients with urinary tract infection before transplantation also

had a higher incidence of urinary tract infection in the period after renal transplantation

In the present study patients in whom E coliuria did *not appear until the period* between transplantation and nephrectomy were not particularly liable to *E coli* in the nephrectomy specimens. In these patients, bacteriuria therefore presumably represented a urinary tract infection (probably as an effect of the immunosuppressive treatment) and not pyelonephritis

Before transplantation, bacteriuria in general did *not predispose to bacteriuria after* the transplantation or to bacterial infection in the kidneys. From this, it seems reasonable to assume that bacteriuria with species other than *E coli* did not have the same tendency to recur or to cause renal infection

The incidence of bacteriuria *after* transplantation (75 per cent) corresponds to that in several other renal transplantation series, in which the incidence of urinary tract infection has shown a range from about 40 per cent to 88 per cent (1 4 5 7 8 9) The relatively high incidence of bacteriuria *before* transplantation (here about 34 per cent) in connection with the tendency of *F coli* infections to recur after transplantation and *to involve the kidneys*, makes it desirable to differentiate between urinary tract infection and pyelonephritis, which however is a problem as the relation of bacteriuria to renal disease is far from being clarified (3 6) Recently the immunofluorescence technique has been used in the detection of antibody coated bacteria in the urine sediment from patients with urinary tract infection, and a positive correlation has been found between the presence of antibody-coated bacteria and the localization of bacteria in the kidneys of patients with urinary tract *infection of un*known localization (2 10 11) This approach seems potentially useful in the differentiation between renal infection and lower urinary tract infection

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This study was supported by a grant from the *Danish Medical Research Council.*

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Acta path. microbiol. scand. Sect. B, 85 455–461 1977

ANTIBODIES AGAINST *E COLI* O ANTIGENS AND COMMON ENTEROBACTERIAL ANTIGEN IN KIDNEY-TRANSPLANT RECIPIENTS

Comparison of Antibody Findings with Evidence of Urinary Tract Infection

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Thomsen, O. F & Hjort, T Antibodies against *E. coli* 0-antigens and common enterobacterial antigen in kidney-transplant recipients. Comparison of antibody findings with evidence of urinary tract infection. Acta path. microbiol. scand. Sect. B, 85 455–461 1977

Using indirect haemagglutination, sera from 45 patients who had undergone renal transplantation were examined for 0-antibodies against 10 selected uropathogenic *E coli* strains, and against common enterobacterial antigen (anti-CA) Bilateral nephrectomy was carried out, usually less than three months after transplantation, and serum from the time of nephrectomy was examined The aim was to correlate antibody findings with evidence of urinary tract infection (UTI) as assessed by significant bacteriuria or growth of bacteria from the nephrectomy specimens All the sera contained antibodies against several of the *E. coli* strains, mostly at titres ≦ 160 but titres up to 5120 occurred. No statistically significant correlation was found between the occurrence of high titres of type-specific 0-antibodies to *E. coli* and evidence of *E coli* UTI In contrast, anti-CA—present in the sera of 19 patients, mostly in titres of 10-40—occurred with significantly increased frequency in patients with E. coliuria before transplantation and in patients with growth of *E. coli* from the nephrectomy specimens. It is concluded that anti-CA—even in low titres—appeared to give a better serological parameter of *E coli* UTI than screening for antibodies against the selected uropathogenic *E. coli* strains.

Key words Antibody *E coli* 0-antigen common enterobacterial antigen kidney-transplant recipients urinary tract infection pyelonephritis.

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Received 10 77 Accepted 30 vii.77

Antibodies against *E coli* 0-antigens can be demonstrated in patients with urinary tract infections (UTI) including patients with pyelonephritis, and it is generally assumed that high antibody titres against *E. coli* isolated from the patients urine indicate infection of the renal parenchyma. Antibody against the common enterobacterial antigen (CA) an antigen shared by most strains of Enterobacteriaceae (8) has also been detected and, by some authors, been claimed to indicate a renal bacterial infection (pyelonephritis) (7 12, 15) whereas others have been unable to confirm this (2, 4)

In an investigation of the renal pathology in patients undergoing renal transplantation,

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it was found that many of the patients had been suffering from UTI both before transplantation and during the period between transplantation and nephrectomy (In most cases bilateral nephrectomy was carried out less than 3 months after transplantation) The bacterial infections were not confined to cases of pyelonephritis, but also occurred in other categories of disease, e.g., in analgesic nephropathy congenital renal disease and in cases of unclassifiable nephropathy (14)

As antibodies against *E. coli* antigens have apparently not been studied in patients undergoing renal transplantation the aims of this study were to examine (1) whether antibodies against the specific O-antigens of the most common uropathogenic *E. coli* strains could be demonstrated in the sera of these patients in spite of the fact that they had been under heavy immunosuppressive therapy (2) to what extent the antibody titres would correspond with evidence of *E. coli* UTI in the patients or with pathological findings, and (3) whether antibody against CA could be demonstrated in the sera, and whether such a finding was of any significance in relation to the occurrence of UTI

MATERIAL AND METHODS

Patients. Sera from 45 consecutive patients who had received a renal transplant were examined. The patients were 30 women and 15 men whose ages ranged from 10 to 63 years and averaged 39 years. Bilateral nephrectomy (N) was carried out after transplantation (T) usually after less than 3 months.

From the time of transplantation, immunosuppressive agents (azathioprine, prednisone) were administered. For details concerning the therapy is referred to (14) Blood samples were withdrawn at the time of N and the sera were stored at -20 C until tested.

Bacteriology A bacterial culture from the nephrectomy specimens was carried out in 45 of the patients as described previously (13)

Pathology Histo-pathological examination of the kidneys revealed eight categories of advanced renal lesions. The morphological criteria for the diagnoses were as described in (13)

Criteria for UTI In this context, UTI was considered to be present (1) when significant bacteriuria could be documented (based on informations from the patients records) and/or (2) when bacteria could be cultured from the nephrectomy specimens. Significant bacteriuria was defined as the presence of $\geq 10^5$ bacteria per ml of voided midstream urine. Patients with clinical and anamnestic evidence of UTI were excluded unless significant bacteriuria had been specifically demonstrated.

Serology Antibodies to *E. coli* O-antigens were determined by indirect haemagglutination, essentially as described by *Neter et al.* (9) Sera were inactivated at 56 C for 30 minutes and absorbed with washed sheep erythrocytes (0 1 ml serum + 0 4 ml 4 per cent sheep erythrocytes in phosphate buffered saline (PBS) (pH 7.2) Serial dilutions of the absorbed sera, using PBS with 1 per cent human serum albumin as diluent, were prepared in plastic trays with U-shaped cups by means of a micro-titre system employing 0 025 ml volumes (1 10 1 20 1 10 240) To each dilution was added 0.025 ml of a 1 per cent suspension of sheep erythrocytes coated with *E. coli* O-antigen. The trays were covered and left at room temperature for 2 hours, after which the agglutination patterns could be read. A mixture of the highest serum concentration (1 10) and uncoated sheep erythrocytes served as control for the specificity of the reaction Known positive and negative sera kindly supplied by Dr *F Ørskov* The International Escherichia Centre Statens Seruminstitut, Copenhagen were included in each batch.

Coating of cells with *E. coli* O-antigens were done by mixing a 4 per cent suspension of washed sheep erythrocytes with an equal volume of the O-antigen in optimal dilution previously determined by chessboard titration. The mixture was incubated at 37 C for 1 hour with frequent shaking The cells were finally washed 3 times in PBS with 1 per cent human serum albumin and a 1 per cent suspension of the cells in the same medium was prepared and used immediately

Preparation of E. coli *O-antigens* Known strains of *E. coli* selected to represent the most common uropathogenic O-types (13) (*E. coli* O1 O2 O4 O6 O7 O8, O9 O18, O22, 75 respectively) were grown for 24 hours in 2 l of brain-heart broth The bacteria were harvested by centrifugation at 7500 rev/min for 15 minutes, and suspended in 50 ml of saline. This suspension of the bacteria was then boiled for 2 hours, and the supernatant obtained after centrifugation contained sufficient O-antigen to be used for coating. A similarly prepared extract of *E. coli* O14 was used for the demonstration of antibodies to CA.

Statistical analysis. Fisher's exact test was used. A 2 p-value < 0.05 was considered to indicate statistical significance.

TABLE 1. *Antibody Titres against 10 Uropathogenic E. coli O-serotypes and E. coli O14 (Anti-CA) in 45 Kidney-Transplant Recipient*

Anti *E. coli* titres	No. of patients with titres against serotypes										
	O1	O2	O4	O6	O7	O8	O9	O18	O22	O75	(CA) O14
5120	0	0	0	0	0	0	1	0	0	0	0
2560	1	1	1	0	0	0	0	0	0	0	0
1280	1	0	0	0	0	1	0	0	0	0	0
640	1	3	1	1	0	2	1	1	0	2	1
320	5	3	2	0	1	18	1	1	1	0	0
160	8	4	3	0	0	11	0	6	2	3	0
80	2	11	5	1	5	8	1	4	2	7	0
40	6	9	10	0	7	4	7	10	3	5	3
20	7	4	9	5	5	1	5	7	3	8	1
10	5	10	10	20	8	0	15	12	8	18	14
0	9	0	5	18	19	0	14	3	26	1	26
Not tested	4							1		2	

RESULTS

Indirect haemagglutination tests with the 45 sera yielded antibody titres against the O-antigens of the 11 *E. coli* strains as listed in Table 1. All the sera contained antibodies against several of the *E. coli* strains. Consequently the mere presence of antibodies to *E. coli* did not form a basis for classification of the patients, but the levels of antibodies had to be taken into consideration.

Most of the titres were ≦ 160 but antibody titres up to 5120 occurred. Furthermore, it is seen that the antibody levels varied for the various serotypes. Thus, all patients had antibodies against *E. coli* O2 and O8, many with relatively high titres against these serotypes and against *E. coli* O1 whereas relatively low titres were found against *E. coli* O6, O22 and O14. As titres ≧ 640 have been regarded as representing infection involving the kidneys (6) this value was chosen as one basis for classification of the patients.

Titres ≧ 640 were relatively rare. Only 19 such reactions were found among 11 patients, some of whom had high antibody levels against more than one serotype of *E. coli*. The clinical and pathological findings in this group of 11 patients were compared with the same parameters in the remaining 34 patients in whom maximal titres did not exceed 320.

However since the antibody levels against the different serotypes varied considerably it would seem a more logical approach to take these variations into account in the classification of the patients. In such an attempt, an arbitrary number equal to 10–15 per cent of the patients with the highest titres for each serotype was considered as one group. As indicated by the line in Table 1, 48 "high" titres were recorded in this way among 21 patients. This "high" titre group was then compared with the corresponding "low" titre group of 24 patients in the same way as mentioned above.

Finally a third classification of the patients was based on the occurrence of antibody against *E. coli* O14 (anti-CA). As seen in Table 1, these titres were generally low with only one high titre (640). Therefore, the patients were grouped simply according to the presence or absence of antibody against CA, giving groups of 19 patients with and 26 without anti-CA.

Table 2 ...

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TABLE 2. *Patho-anatomical Lesions and Antibody Findings against* E. coli *O-antigens and CA in 45 Kidney-Transplant Recipients*

Patho-anatomical diagnoses	No. of patients	Titre ≧ 640	"High" titre	+ Anti-CA
Obstructive chronic pyelonephritis	4		2	3
Analgesic nephropathy	13	5	7	8
Congenital renal disease	9	1	4	3
Glomerulonephritis	6	2	4	1
Nephrosclerosis	3	1	2	
Diabetic nephropathy	1			
Unclassifiable chronic nephropathy	8	2	2	4
Sequelae of cortical necrosis	1			
Total	45	11	21	19

cording to the three principles of classification related to the patho-anatomical lesions. Titres ≧ 640 occurred in 5 of 13 patients with analgesic nephropathy. On the other hand, all 4 patients with obstructive chronic pyelonephritis had titres ≦ 320 although bacteriuria after T had been present in three of them. "High" and "low" titres occurred without any marked relation to the pathological diagnoses. Anti-CA was present in most cases of obstructive chronic pyelonephritis and analgesic nephropathy whereas only one of the 6 patients with chronic glomerulonephritis, and none of the 3 patients with nephrosclerosis, had anti-CA.

Serum from the only patient with a high titre of anti CA (640) did also as might be expected react with extracts of all the other *E. coli* strains (in most cases with titres of 640). However as far as the sera with low titres of anti-CA are concerned, there was no apparent correlation with either titres ≧ 640 or "high" titres.

UTI with *E. coli* was recorded in 28 of the 45 patients, either on the basis of E. coli uria (18 cases) growth of *E. coli* from the nephrectomy specimens (1 case) or both findings (9 cases). (In one of the cases of only E. coliuria, staphylococci could be cultured from the nephrectomy specimens.) Ten patients had bacteriuria with organisms other than *E. coli* and in 7 patients bacteriuria was not recorded; the nephrectomy specimens were sterile in the two groups of patients just mentioned. In Table 3 the antibody findings are related to the bacteriological results. Anti-CA was found in 16 of the 28 patients with *E. coli* UTI but only in 3 of the 17 patients with other or no UTI, i.e. significantly more frequently in patients with *E. coli* UTI ($2p = 0.02$). Corresponding, although not significant, dif

TABLE 3. *Antibody Findings Related to the Occurrence of Previous and/or Recent Urinary Tract Infection in 45 Kidney-Transplant Recipients*

	No. of patients	Titre ≧ 640	"High" titre	+ Anti-CA
E. coli UTI	28	8	14	16
UTI with other bacteria	10	2	6	1
No UTI	7	1	1	2
Total	45	11	21	19

TABLE 4 *Antibody Findings Related to E. coli ria befor and fter Transplantation in 45 Kidney-T an plant Recipients*

Occurrence of E. coliur.a	No. of patients	Titre ≧ 640	High" titre	+ Anti-CA
Only before transplantation	1			1
Before transpl. and between transpl. and nephr	10	5	7	9
Only between transpl. and nephr	16	3	7	5
Absent	18	3	7	4
Total	45	11	21	19

ferences were seen for O-antibodies to the uropathogen.c *E coli* strains when titres ≧ 640 were considered. Here, 8 patients with and 3 without *E coli* UTI had such titres (2 p > 0.4) As regards "high titres, no difference was seen.

It should be noted that no evidence of *E. coli* UTI was found in 3 of the 11 patients with titres ≧ 640 against *E coli*. A review of the records of these patients revealed no evidence of either *E. coli* sepsis or *E coli* infect on in extrarenal foci.

E. coliuria occurred in a total of 27 pa tients (In further one patient, as mentioned, *E. coli* could be cultured from the nephrec tomy *specimens, whereas E. coliuria was not* recorded) In order to see whether the antibody findings were related to the duration of E. coliuria the 10 patients in whom E. col. uria was recorded both before T and between T and N were compared with the 16 patients with E. coliuria only between T and N (Table 4) However it should be borne in mind that all patients were treated with immunosuppressive drugs during the period between T and N Consequently the material allows comparison only between cases of prolonged E. coliuria, which had developed before immunosuppression. From Table 4 it is seen, that anti-CA was found in 9 of the 10 patients with E coliuria both before and after T but in 5 of the 16 patients with E. coli uria only between T and N i.e. it was sig nificantly more frequent in the former group (2 p = 0.009) In contrast, the levels of anti bodies against type-specific *E. coli* O-antigens occurred without marked differences in the *two groups of patients.*

Bacterial growth from the nephrectomy specimens can be regarded as a strong in dication of infection involving the renal parenchyma, and in the present study *E coli* could be cultured from the nephrectomy specimens of 10 patients. In order to see

TABLE 5 *Antibody Findings R at d to E.* coli *in N ph ectomy Specimens the 43 Patients in whom Growth Cultur was Carried out*

Growth of *E. coli* from nephrectomy specimens	No. of patients		Titre ≧ 640	"High titre	+ Anti-CA
Present	10		5	6	8
Absent	33	E. coliuria 16	3	8	7
		Other bacteriuria 10	2	6	1
		No bacteriuria 7	1	1	2
Total	43		11	21	18

whether the presence of bacteria in the renal tissue would influence the antibody findings, the patients with and without growth of *E coli* from the nephrectomy specimens were compared (Table 5) Here only the 43 patients in whom this examination had been carried out have been included. Among the 10 patients who had *E coli* in their nephrectomy specimens, 8 had anti-CA i.e. it was significantly more frequent than among the 33 patients without *E coli* in the nephrectomy specimens, of whom 10 had anti-CA ($2p = 0.015$) Corresponding although not significant differences were seen for antibody titres ≧ 640 against the uropathogenic *E coli* strains.

DISCUSSION

Antibodies against one or more of the O antigens of 10 common uropathogenic strains of *E coli* could be demonstrated in all the patients, in spite of the immunosuppressive therapy However the levels of titres varied considerably for the various O-serotypes, as was also found by *Ehrenkranz & Carter* (5) who in their study of patients with UTI obtained higher antibody titres in patients infected with certain O-serotypes than in those infected with others.

Anti-CA occurred most frequently in patients in whom *E coli* UTI had been present. In contrast a corresponding correlation was not found for O antibody titres ≧ 640 against the uropathogenic *E coli* strains. This might be due to the fact that our panel of *E. coli* O-antigens did not cover all *E coli* However the use of these antigens was based on the findings of several investigators viz. that these relatively few O serotypes cause up to two thirds of UTIs with *E coli* (13)

Moreover anti-CA was found most frequently among patients in whom E. coliuria was observed already before transplantation

As regards antibodies against the uropathogenic serotypes, no difference was seen between patients with E. coliuria before T and those with E coliuria before and after T However such antibodies occurred more frequently in each of these groups than in the small group of patients without bacteriuria This might indicate that an immune response may be elicited during immunosuppression in these patients, as has also been found for antibodies to other micro-organisms, such as cytomegalovirus (3) Further studies with several samples from each patient will be needed to clarify this.

With regard to the relation of antibody titres to renal involvement of a bacterial infection several investigations indicate that involvement of the renal parenchyma gives rise to higher titres of antibodies to *E. coli* than lower UTI usually does (1 10) Most frequently titres of ≧ 320 (11) or ≧ 640 (6) as assessed by the indirect haemagglutination test, have been regarded as reflecting a renal infection. In this study the presence of bacteria in the nephrectomy specimens could be regarded as an indication of renal involvement. Here, anti-CA was correlated with growth of *E coli* from the nephrectomy specimens, whereas a significant correlation was not found for high antibody titres to the uropathogenic *E. coli* strains. The rare finding of anti-CA in patients with bacteriuria other than E coliuria, may be connected with the fact that bacteria could not be cultured from the nephrectomy specimens in these patients.

Some investigators have claimed that a determination of antibody against CA is of value in the diagnosis of chronic pyelonephritis (7 12, 15) On the other hand, in studies of antibodies in acute UTI and pyelonephritis other investigators have denied any diagnostic value of the measurement of anti CA (2 4) In the last mentioned studies the main objections were that titres against CA were low rarely exceeding 100 and that no correlation was seen with haemagglutinin levels in other serogroups studied.

The finding of antibodies in the patients of the present study must obviously be seen in the light of the modified state of resistance to bacterial infection and ability to elicit immune responses brought about by the immunosuppressive therapy Nevertheless,

the results indicate that the detection of antibody against CA, even when occurring in low titres, appears to yield a better serological parameter of *E. coli* infection—particularly with renal involvement—than a screening for antibodies against the uropathogenic *E. coli* strains used in this study. However anti-CA can apparently also be found in patients without evidence of UTI, and the mere demonstration of anti-CA therefore does not in itself allow conclusions.

This study was supported by a grant from the *Danish Medical Research Council.*

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Acta path microbiol scand. Sect. B 85 462–465 1977

BRIEF REPORTS

EXPERIMENTAL MYCOPLASMAL PNEUMONIA IN DOGS ELECTRON MICROSCOPY OF INFECTED TISSUE

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Rosendal, S & Vinther O Experimental mycoplasmal pneumonia in dogs Electron microscopy of infected tissue Acta path. microbiol. scand. Sect B 85 462–465 1977

Thin sections of lung tissue from dogs with pneumonia induced by endobronchial inoculation of Mycoplasma cynos were examined by electron microscopy Mycoplasmas were observed extracellularly in lumen of bronchus and in alveoli in the earlier stages of the infection. The infection also resulted in degenerative changes in the bronchial epithelial cells, including in particular destruction and loss of cilia.

Key words Mycoplasma cynos mycoplasmal pneumonia electron microscopy

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Received 9 vii 77 Accepted 14.x.77

Pneumonia was induced by endobronchial inoculation of *Mycoplasma cynos* strain D19 into 1-week-old dogs (4) The present communication gives the results of electron microscopic examination of infected tissue from three dogs killed at regular intervals after inoculation. Histologically the lesions induced were characterized by severe inflammation of bronchi and adjacent respiratory tissue. At post-mortem large numbers of mycoplasmas were isolated from affected tissue of the two dogs killed first whereas none were recovered from the last one.

Material and Methods

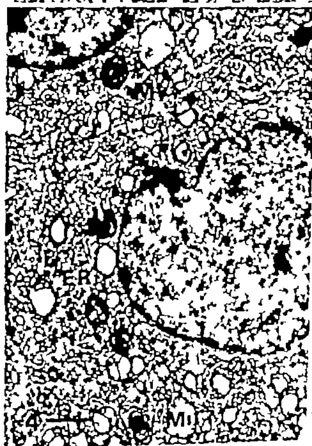
The dogs, D23 D24 and D25 were killed with an overdose of barbiturate 2, 3 and 4 weeks after inoculation, and tissue was removed immediately and cut into blocks about 1 × 1 × 2 mm. These blocks were fixed by immersion in 2 per cent glutaraldehyde in 0 1 M sodium cacodylate buffer pH 7.2 for 4 h at room temperature. After a brief wash in cacodylate buffer containing 5 per cent sucrose, the blocks were further fixed in 1 per cent OsO_4 in Michaelis buffer pH 7.2 for 1 h at room temperature and subsequently stained for 15 min in 2 per cent uranyl acetate in 70 per cent ethanol. After acetone dehydration and embedding in Vestopal-W ultrathin sections were obtained with an LKB Ultrotome III microtome post-stained with magnesium uranyl acetate and lead citrate and examined in a JEOL JEM 100 B electron microscope.

Based on microscopical examination of toluidine blue (0 1 per cent) stained sections, representative areas of bronchial and alveolar inflammatory lesions were chosen for electron microscopy For

Fig 1 D23 Macrophage in lumen of bronchus. Several mycoplasmas (M) are present extracellularly One mycoplasma (Mp) seems to be in the process of being phagocytosed. Lysosomes (L) Possible phagosome (P) × 41,000 Bar represents 250 nm.

Fig 2 D23 Macrophage containing phagosome (P) in the lumen of a bronchus Mycoplasma structures (M) are seen in the phagosome and extracellularly In addition, there is membrane debris (Me) in the phagosome The macrophage surface is irregular with many pseudopods (Ps) × 61,000 Bar represents 250 nm.

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comparison purposes, lung tissue from areas without histologically visible lesions was also included in the study.

Results

D23. The epithelial cells of the inflamed bronchi were swollen and vacuolated. Cilia were fractured and deformed and were less in number compared to the normal bronchus. Membranous debris and desquamated epithelial cells were seen in the lumen together with neutrophils and macrophages. Mycoplasmas were observed in different areas of the lumen. In Fig. 1 several mycoplasmas, characterized by their densely packed ribosomes and their triple-layered limiting membrane, are located near a macrophage which is swollen and surrounded by a damaged or broken plasma membrane. The cytoplasmic matrix of the macrophage is lucent and the cytoplasmic organelles partly disorganized. One organism, possibly in an early stage of being phagocytosed, can be seen. Macrophages with phagosomes containing mycoplasma-like structures, as well as unidentifiable membrane debris, were also encountered (Fig. 2).

In sections of alveoli, the lining cells were swollen and vacuolated. Alveolar spaces contained neutrophils, macrophages, and unidentifiable debris. Mycoplasmas were also observed in the alveoli.

D24. The epithelial cells of the bronchi were very swollen and appeared to be vacuolated due to pronounced distention of the endoplasmic reticulum. The mitochondria were also swollen. However, the most striking feature was the severe destruction of cilia which, in most cases could only be identified by their remaining basal bodies. Membrane and cilia debris were observed in the lumen together with a few mycoplasmas (Fig. 3). Several plasma cells and macrophages were present in the peribronchial area.

Degenerated neutrophils and macrophages were observed in the alveoli, together with unidentifiable debris. Mycoplasmas could not be demonstrated in the alveoli of this dog. Degenerative changes in bronchial and alveolar epithelium were not observed in tissue without visible lesions (Fig. 5).

D25. Only very few basal bodies were left of the cilia of the epithelial cells, otherwise the same changes were observed as seen in D24.

The alveoli were filled with more or less degenerated cells and membrane debris. Fig. 4 shows alveolar macrophages. The endoplasmic reticulum of these cells is distended and their mitochondria are swollen. In addition myelin-like structures are very prominent in the cytoplasm. Mycoplasmas were not seen, either in the bronchus or in the alveoli.

Discussion

This paper reports on the electron microscopical demonstration of mycoplasmas in dogs after endobronchial inoculation of the animals with *M. cynos*. The mycoplasmas were easily recognized on the basis of their characteristic morphology as demonstrated previously in laboratory cultures of *M. cynos* (3). The organisms were mainly located extracellularly but some could also be found in phagosomes of macrophages and neutrophilic granulocytes.

The histopathologic characteristics (*Rosendal*, unpublished results (4)) were, in general, confirmed by electron microscopy. One feature which deserves special attention is the degenerative changes in the bronchial epithelial cells, including the destruction and loss of cilia. These changes are probably caused by the mycoplasmas and not by inadequate fixation, since the ultrastructure of lung tissue from areas without visible lesions was generally well preserved. The bronchial changes resemble closely those observed in rats inoculated with *Mycoplasma pulmonis* (2) and in hamsters infected with *Mycoplasma pneumoniae* (1).

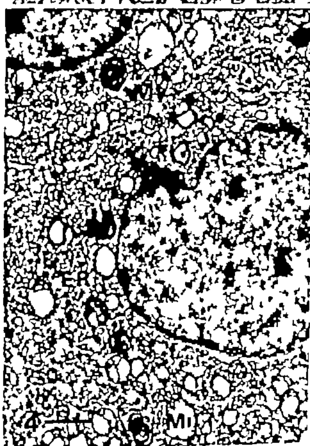
Fig. 3. D24. Section of bronchus. The epithelial cells are swollen and show distended endoplasmic reticulum (ER) and swollen mitochondria (Mi). Mycoplasmas (M), cilia (C) and membrane debris (Me) are present in the lumen. Basal bodies (B) of cilia can be seen at the epithelial surface. × 12,000. Bar represents 1 μm.

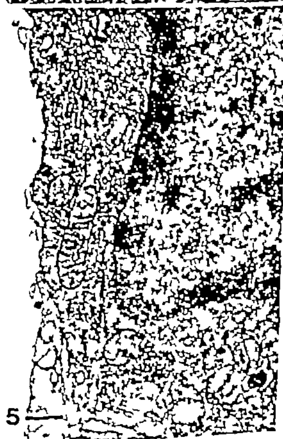
Fig. 4. D25. Alveolar macrophages. Degeneration of these cells is evident from the marginated and clumped chromatin, the distended endoplasmic reticulum (ER), the swollen mitochondria (Mi) and the prominent myelin figures (My) present in their cytoplasm. × 10,000. Bar represents 1 μm.

Fig. 5. D24. Alveolar epithelial cell from an area without histologically visible lesions. The ultrastructure of the cell is well preserved. Round and elongated mitochondria and flat endoplasmic reticulum are observed in the cytoplasm of the cell. × 33,000. Bar represents 250 nm.

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ER
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This paper reports on the electron microscopical demonstration of mycoplasmas in dogs after endobronchial inoculation of the animals with *M. cynos*. The mycoplasmas were easily recognized on the basis of their characteristic morphology as demonstrated previously in laboratory cultures of *M. cynos* (3). The organisms were mainly located extracellularly but some could also be found in phagosomes of macrophages and neutrophilic granulocytes.

The histopathologic characteristics (*Rosendal*, unpublished results (4)) were, in general, confirmed by electron microscopy. One feature which deserves special attention is the degenerative changes in the bronchial epithelial cells, including the destruction and loss of cilia. These changes are probably caused by the mycoplasmas and not by inadequate fixation, since the ultrastructure of lung tissue from areas without visible lesions was generally well preserved. The bronchial changes resemble closely those observed in rats inoculated with *Mycoplasma pulmonis* (2) and in hamsters infected with *Mycoplasma pneumoniae* (1).

Fig. 3. D24. Section of bronchus. The epithelial cells are swollen and show distended endoplasmic reticulum (ER) and swollen mitochondria (M). Mycoplasmas (M), cilia (C) and membrane debris (Me) are present in the lumen. Basal bodies (B) of cilia can be seen at the epithelial surface. × 12,000. Bar represents 1 µm.

Fig. 4. D25. Alveolar macrophages. Degeneration of these cells is evident from the marginated and clumped chromatin, the distended endoplasmic reticulum (ER), the swollen mitochondria (Mi) and the prominent myelin figures (My) present in the cytoplasm. × 10,000. Bar represents 1 µm.

Fig. 5. D24. Alveolar epithelial cell from an area without histologically visible lesions. The ultrastructure of the cell is well preserved. Round and elongated mitochondria and flat endoplasmic reticulum are observed in the cytoplasm of the cell. × 33,000. Bar represents 250 nm.

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Acta path. microbiol. scand. Sect. B 85 466–468 1977

TITRATION OF HERPES SIMPLEX VIRUS ANTIBODIES IN HUMAN SERA BY THE ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

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Vestergaard, B F., Grauballe P C. & Spanggaard, H Titration of herpes simplex virus antibodies in human sera by the enzyme-linked immunosorbent assay (ELISA) Acta path. microbiol scand. Sect. B, 85 466–468 1977

100 sera from healthy adults were titrated simultaneously for herpes simplex virus (HSV) antibodies by ELISA and neutralization. The ELISA was performed on microtitre plates where approximately 100 ng of detergent solubilized and chromatographically purified HSV glycoproteins was bound covalently to the plastic bottom of each well The optical density (OD) values obtained by the use of the peroxidase-1 2 phenylendiammindihydrochloride system showed good correlation with the neutralizing antibody titres. Sera with very low neutralizing titres were clearly positive in ELISA

Key words Herpes simplex virus antibodies ELISA.

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Received 15.x.77 Accepted 15.x.77

In 1972, *Engvall & Perlmann* described an enzyme-linked immunosorbent assay (ELISA) for the quantification of antibodies and antigens (2) The assay combines specificity with a high degree of sensitivity and has been successfully adapted to different viral antigen antibody systems (5 10 11) We needed a simple routine test for the titration of herpes simplex virus (HSV) antibodies and the following describes the use of detergent solubilized partially purified HSV glycoproteins in ELISA.

Materials and Methods

Antigen Crude antigen was prepared from Triton X 100 solubilized HSV type 1 infected rabbit cornea cells, as described previously (6) The solubilized HSV proteins were then purified by ion-exchange chromatography and the glycoprotein preparation previously designated fraction I (8) was used as antigen in the ELISA. Control antigen from uninfected cells was made in a similar manner

Sera. One hundred sera from normal healthy adults aged 20 to 50 years, representing both sexes, were tested in ELISA at dilutions 1 10 1 100 and 1 1000 One negative and one positive reference serum were used throughout the study

ELISA The microtitration plate system (Cooke M220-25A) was used. *Pre-coating* 100 µl of 0 1 per cent bovine albumin (fraction V Sigma No A-4503) in distilled water was added to each well and allowed to dry out at room temperature. *Antigen coating* 100 µl of 0.25 per cent glutaraldehyde (25 per cent glutaraldehyde Fluka AG pract 49630) in phosphate-buffered saline (PBS) adjusted to pH 7 0 with NaOH was added to each well After 30 min at room temperature the wells were emptied and washed three times in 150 µl of distilled water 100 µl of a 1 200 dilution of antigen was then added to each well and allowed to dry out at room temperature followed by washing three times, as described above *Immune reaction* Human sera and peroxidase con

jugated rabbit anti-human-IgG (P 1090 DAKO-immunoglobulins, Copenhagen, Denmark) were diluted in 0.5 per cent bovine albumin with 0.05 per cent Tween 20 (Art. 822184 Merck) in PBS. 100 µl of human sera dilutions was added to each well and incubated for 1 hour at 37 C, followed by washing three times (150 µl of 0.05 per cent Tween 20 in PBSS). 100 µl of the enzyme conjugate diluted 1:200 was then added, followed by incubation and washing, as described above. *Enzyme reaction.* 55 mg of 1,2 phenylendiamindihydrochloride (Fluka AG, purum) was dissolved in 100 ml of an 0.04 M Tris-HCl buffer pH 7.6 with 0.9 per cent NaCl. Just before use 50 µl of 30 per cent H_2O_2 was added and 100 µl of the reaction mixture was placed in each well. The reaction proceeded for 5 min at room temperature and was stopped by the addition of 50 µl of 0.1 N H_2SO_4. The contents of each well were transferred to 0.5 ml of 0.1 N H_2SO and optical density (OD) as read in a Zeiss spectrophotometer at 450 nm.

Immunoelectrophoresis. Countercurrent electrophoresis was performed according to the method described by *Grandballe et al.* (3)

Neutralization. Plaque reduction multiplicity assays as carried out as described earlier (9)

Statistics. The correlation between ELISA OD values and neutralizing titres was calculated by Spearman rank correlation coefficient.

Results and Discussion

Fig. 1 shows that the HSV antigen preparation diluted 1:1000 gas positive reaction, while the control antigen did not react at dilution 1:20. Immunoelectrophoretic studies of the HSV antigen (8, 9) has shown that the preparation contained two major HSV type-common glycoproteins and two HSV type 1-specific glycoproteins. Countercurrent immunoelectrophoretic titration of the HSV antigen preparation against different dilutions of polyspecific rabbit antiserum against HSV type 1 showed that 15 µl of an antigen dilution of 1:100 gas just visible precipitates after Coomassie Brilliant Blue staining. The amount of specific antigen at this dilution should be somewhere between 10 and 100 ng, according to quantitative immunoelectrophoretic studies (1). This means that the ELISA was able to detect from 6 to 60 ng of HSV protein, and the working dilution of 1:200 contained from 50 to 500 ng.

Fig. 2 shows that he positive reference serum diluted 1:10,000 reacted positively while the negative reference serum was negative at 1:10. The OD values of the positive reference serum at 1:10 to 1:10,000 follow straight line. Similarly the OD values at 1:10, 1:100 and 1:1000 of the positive test sera followed straight lines running parallel with the line of the reference serum. The titre

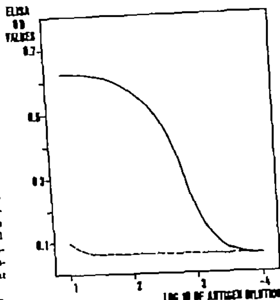


Fig. 1. ELISA OD values of two-fold dilutions of HSV antigen and control antigen (1:10 to 1:10,240) with positive reference serum 1:10. HSV antigen ——— Control antigen - - - - -

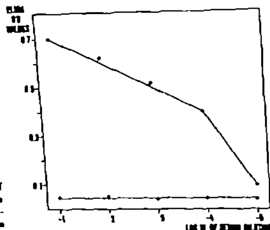


Fig. 2. ELISA OD values of ten-fold dilutions of positive and negative reference sera with HSV antigen at 1:200. Positive serum ——— Negative serum. - - -

of a test serum can thus be expressed as the difference in dilution between reference serum and test serum giving the same OD value (*Leinikki* in press). However this method requires the testing of at least three different dilutions of each serum. The titre can be expressed more simply as the OD value

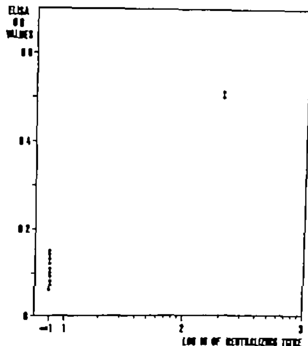


Fig 3 ELISA OD values and neutralizing antibody titres of 100 sera. The OD values were measured at serum dilution 1 100 and the neutralizing titres represent the dilution causing a 90 per cent plaque reduction

at just one dilution, or as the percentage of the OD value of a reference serum at a single dilution (4)

In Fig 3 we have chosen to present the OD values found at dilution 1 100 The range of the OD values of the positive reference serum from plate to plate during the test period was 0.58 to 0 66 Fig 3 shows the very good correlation between OD values and viral neutralizing titres. The correlation coefficient was calculated at 0 91 corresponding to a T value of 8 93 or $P < 0.001$ The few "negative" sera with OD values above 0 1 did contain very small amounts of HSV neutralizing antibodies as judged by the plaque count, but they were unable to produce a 90 per cent plaque reduction at dilution 1 10 The sensitivity of the ELISA was demonstrated by the high OD values found with sera containing low titre neutralizing antibodies (1 10 to 1 100)

Finally we wish to emphasize two points 1) In the present work, unlike other published ELISA studies, we have used detergent-solubilized antigens that could be characterized by quantitative immunoelectrophoretic methods (8,9) and 2) Covalent binding of the antigens to the plastic permits extensive washings which lower the background reactivity without any reduction in the specific OD values.

This work was supported by a grant from the Danish Cancer Society

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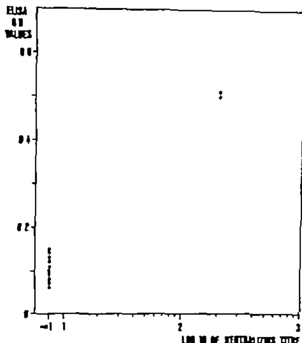


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ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
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Vol. 85 B. Fasc. 6. 1977

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